



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b>  <b>A61F 13/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 92/19195</b>  <b>(43) International Publication Date:</b> 12 November 1992 (12.11.92)
<b>(21) International Application Number:</b> PCT/US92/03327 <b>(22) International Filing Date:</b> 22 April 1992 (22.04.92) <b>(30) Priority data:</b> 692,403 25 April 1991 (25.04.91) US <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 692,403 (CIP) Filed on 25 April 1991 (25.04.91) <b>(71) Applicant (for all designated States except US):</b> BROWN UNIVERSITY RESEARCH FOUNDATION [US/US]; Box 1949, Graduate Center, 3rd Floor, Brown University, 42 Charlesfield Street, Providence, RI 02912 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> DIONNE, Keith, E. [US/US]; 35 Fairfield Street, Rehoboth, MA 02769 (US). EMERICH, Dwaine, F. [US/US]; 13 Eleventh Street, Providence, RI 02908 (US). HOFFMAN, Diane [US/US]; 33 Belmont Street, Apartment 2, Somerville, MA 02143 (US). SCHARP, David, W. [US/US]; 1129 Dunston Drive, St. Louis, MO 63146 (US). LACY, Paul, E. [US/US]; 63 Marshall Place, Webster Groves, MO 63119 (US). AEBISCHER, Patrick [US/US]; 7 Cheshire Drive, Barrington, RI 02806 (US). SANBERG, Paul, R. [US/US]; 11751 Pilot Country Drive, Spring Hill, FL 34610 (US). CHRISTENSON, Lisa [US/US]; 120 Progress		Street, Pawtucket, RI 02860 (US). HEGRE, Orion, D. [US/US]; 28 Whitehouse Drive, Chepachet, RI 02814 (US). VASCONCELLOS, Alfred, V. [US/US]; 766 Lat-en Knight Road, Cranston, RI 02918 (US). LYSAGHT, Michael, J. [US/US]; 16 River Run, East Greenwich, RI 02818 (US). GENTILE, Frank, T. [US/US]; 57 Lawn Avenue, Warwick, RI 02888 (US). <b>(74) Agents:</b> BREZNER, David, J. et al.; Flehr, Hohbach, Test, Albritton & Herbert, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US). <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent), US. <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> IMPLANTABLE BIOCOMPATIBLE IMMUNOISOLATORY VEHICLE FOR DELIVERY OF SELECTED THERAPEUTIC PRODUCTS		
<b>(57) Abstract</b>  <p>An immunoisulatory vehicle for the implantation into an individual of cells which produce a needed product or provide a needed metabolic function. The vehicle is comprised of a core region containing isolated cells and materials sufficient to maintain the cells, and a permselective, biocompatible, peripheral region free of the isolated cells, which immunoisolates the core yet provides for the delivery of the secreted product or metabolic function to the individual. The vehicle is particularly well-suited to delivery of insulin from immunoisolated islets of Langerhans, and can also be used advantageously for delivery of high molecular weight products, such as products larger than IgG. A method of making a biocompatible, immunoisulatory implantable vehicle, consisting in a first embodiment of a coextrusion process, and in a second embodiment of a stepwise process. A method for isolating cells within a biocompatible, immunoisulatory implantable vehicle, which protects the isolated cells from attack by the immune system of an individual in whom the vehicle is implanted. A method of providing a needed biological product or metabolic function to an individual, comprising implanting into the individual an immunoisulatory vehicle containing isolated cells which produce the product or provide the metabolic function.</p>		

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

**IMPLANTABLE BIOCOMPATIBLE IMMUNOISOLATORY VEHICLE  
FOR DELIVERY OF SELECTED THERAPEUTIC PRODUCTS**

Related Application

This is a continuation-in-part application under 35 USC  
5 120 of application U.S.S.N. 07/692,403, filed 25 April  
1991.

Background

Many clinical conditions, deficiencies, and disease states  
can be remedied or alleviated by supplying to the patient  
10 a factor or factors produced by living cells or removing  
from the patient deleterious factors which are metabolized  
by living cells. In many cases, these factors can restore  
or compensate for the impairment or loss of organ or  
tissue function. Examples of disease or deficiency states  
15 whose etiologies include loss of secretory organ or tissue  
function include (a) diabetes, wherein the production of  
insulin by pancreatic islets of Langerhans is impaired or  
lost; (b) hypoparathyroidism, wherein the loss of  
production of parathyroid hormone causes serum calcium  
20 levels to drop, resulting in severe muscular tetany; (c)  
Parkinsonism, wherein dopamine production is diminished;  
and (d) anemia, which is characterized by the loss of  
production of red blood cells secondary to a deficiency in  
erythropoietin. The impairment or loss of organ or tissue  
25 function may result in the loss of additional metabolic  
functions. For example, in fulminant hepatic failure,  
liver tissue is rendered incapable of removing toxins,  
removing the products of cell metabolism, and secreting

-2-

essential products, such as albumin and Factor VIII.  
Bontempo, F.A., et al., (1987) Blood 69:1721-1724.

In other cases, these factors are biological response  
modifiers, such as lymphokines or cytokines, which enhance  
5 the patient's immune system or act as anti-inflammatory  
agents. These can be particularly useful in individuals  
with a chronic parasitic or infectious disease, and may  
also be useful for the treatment of certain cancers. It  
may also be desirable to supply trophic factors to a  
10 patient, such as nerve growth factor or insulin-like  
growth factor-one or -two (IGF1 or IGF2).

In many disease or deficiency states, the affected organ  
or tissue is one which normally functions in a manner  
responsive to fluctuations in the levels of specific  
15 metabolites, thereby maintaining homeostasis. For  
example, the parathyroid gland normally modulates  
production of parathyroid hormone (PTH) in response to  
fluctuations in serum calcium. Similarly,  $\beta$  cells in the  
pancreatic islets of Langerhans normally modulate  
20 production of insulin in response to fluctuations in serum  
glucose. Traditional therapeutic approaches to the  
treatment of such diseases cannot compensate for the  
responsiveness of the normal tissue to these fluctuations.  
For example, an accepted treatment for diabetes includes  
25 daily injections of insulin. This regimen cannot  
compensate for the rapid, transient fluctuations in serum  
glucose levels produced by, for example, strenuous  
exercise. Failure to provide such compensation may lead  
to complications of the disease state; this is  
30 particularly true in diabetes. Jarret, R.J. and Keen J.,  
(1976) Lancet(2):1009-1012.

Accordingly, many investigators have attempted to  
reconstitute organ or tissue function by transplanting  
whole organs, organ tissue, or cells which provide



-3-

secreted products or affect metabolic functions. Moreover, transplantation can provide dramatic benefits but is limited in its application by the relatively small number of organs suitable and available for grafting. In general, the patient must be immunosuppressed in order to avert immunological rejection of the transplant, which results in loss of transplant function and eventual necrosis of the transplanted tissue or cells. In many cases, the transplant must remain functional for a long period of time, even for the remainder of the patient's lifetime. It is both undesirable and expensive to maintain a patient in an immunosuppressed state for a substantial period of time.

A desirable alternative to such transplantation procedures is the implantation of cells or tissues within a physical barrier which will allow diffusion of nutrients, waste materials, and secreted products, but block the cellular and molecular effectors of immunological rejection. A variety of devices which protect tissues or cells producing a selected product from the immune system have been explored. These include extravascular diffusion chambers, intravascular diffusion chambers, intravascular ultrafiltration chambers, and implantation of microencapsulated cells. Scharp, D.W., et al. (1984) World J. Surg. 8:221-9. These devices were envisioned as providing a significant advance in the field of transplantation, as they would alleviate the need to maintain the patient in an immunosuppressed state, and would thereby allow many more patients to receive restorative or otherwise beneficial transplants by allowing the use of donor cells or tissue which could not have been used with the conventional transplantation techniques. However, none of these approaches have been satisfactory for providing long-term transplant function. A method of delivering appropriate quantities of needed substances, such as enzymes and hormones, or of providing

-4-

other needed metabolic functions, for an extended period of time is still unavailable and would be very advantageous to those in need of long-term treatment.

#### Summary of the Invention

5 This invention relates to a biocompatible, immunoisulatory, implantable vehicle. The instant vehicle is suitable for isolating biologically active cells or substances from the body's protective mechanisms following implantation into an individual. The instant vehicle is  
10 comprised of (a) a core which contains isolated cells, either suspended in a liquid medium or immobilized within a hydrogel matrix, and (b) a surrounding or peripheral region ("jacket") of permselective matrix or membrane which does not contain isolated cells, which is  
15 biocompatible, and which is sufficient to protect the isolated cells in the core from immunological attack.

Herein, the term "individual" refers to a human or an animal subject.

As defined herein, the term "dual matrix vehicles" refers  
20 to such vehicles with a cell-containing core and an external jacket free of cells. In one embodiment, the matrix core is formed of a hydrogel which is cross-linked to a hydrogel jacket, suitably in the form of a rod or other shape. The hydrogel jacket may be formed  
25 independently as a sheath around the matrix without cross-linking. The hydrogel core is not necessarily linked to the outer jacket by means of opposite ionic charges. In another embodiment, the external jacket is formed of a thermoplastic material which is not linked to the core  
30 matrix by chemical bonding.

As used herein, unless otherwise specified, the term "cells" means cells in any form, including but not limited

-5-

to cells retained in tissue, cell clusters, and individually isolated cells, unless otherwise specified.

The core of the immunoisulatory vehicle is constructed to provide a suitable local environment for the particular  
5 cells isolated therein. In some embodiments, the core comprises a liquid medium sufficient to maintain the cells. Liquid cores are particularly suitable for maintaining transformed cells, such as PC12 cells. In  
10 other embodiments, the core comprises a gel matrix which immobilizes and distributes the cells, thereby reducing the formation of dense cellular agglomerations. The gel matrix may be composed of hydrogel or extracellular matrix components.

Cores made of a hydrogel matrix are particularly suitable  
15 for maintaining cells or tissues which tend to form agglomerates, such as the cells in islets of Langerhans, or adrenal chromaffin cells. The matrix should be of sufficient viscosity to maintain cell dispersion within the matrix. Optionally, the core of the instant vehicle  
20 can contain substances which support or promote the function of the isolated cells. These substances include natural or synthetic nutrient sources, extracellular matrix (ECM) components, growth factors or growth regulatory substances, or a population of feeder or  
25 accessory cells or O<sub>2</sub> carriers such as hemoglobins and fluorocarbons.

In previously existing devices, the core and jacket were linked via ionic bonds between oppositely charged polymers in one of two ways. (1) The devices of Rha (U.S. Patent  
30 No; 4,744, 933) were constructed of a charged inner core material and an outer jacket material of the opposite charge. (2) The devices of Lim and Sun (U.S. Patent Nos: 4,352,833 and 4,409,331) included an intermediate layer of poly-L-lysine (PLL), which is positively charged, to link

-6-

the negatively charged core with the negatively charged jacket material. The elimination of a PLL layer is advantageous in that PLL is believed to be fibrogenic in the host. PLL may also have unwanted growth effects for some cells. Also, the jacket of the device of the invention can be controlled for permselectivity better than those made with PLL.

The jacket of the instant vehicle is made of a material which may be the same as that of the core or may be different. In either case, the material used results in a surrounding or peripheral region which is permselective, biocompatible and immunoisulatory.

The jacket may be formed freely around the core without chemical bonding or, alternatively, the jacket may be directly cross-linked to the core matrix. In either case, formation of the vehicle of the present invention does not require polymers of opposite charge to the core being present in an interfacial layer or in the jacket.

Preferably, the core and external jacket form an interface free of "ionic bonding" between oppositely charged polymers and free of an intermediate layer of the type used in prior art microcapsules. Ionic bonding refers to an ionic interaction of a core of one charge (positive or negative) and the jacket (or an intermediate layer) of opposite charge.

The jacket allows passage of substances up to a predetermined size, but prevents the passage of larger substances. More specifically, the surrounding or peripheral region is produced in such a manner that it has pores or voids of a predetermined range of sizes; as a result, the vehicle is permselective. The molecular weight cutoff (MWCO) selected for a particular vehicle will be determined in part by the type and extent of immunological rejection it is anticipated will be

-7-

encountered after the vehicle is implanted and in part by the molecular size of the largest substance to be allowed to pass into and/or out of the vehicle. For example, materials can be used to form permselective membranes or hydrogel matrices which allow passage of molecules up to about the size of C1q, a component of complement (about 400 kD), a protein required for the assembly of the cytolytic complement attack complex. In this instance, substances smaller than C1q can pass freely. It is also possible to form permselective matrices or membranes which allow passage of molecules up to about the size of immunoglobulin G (about 150 kD) and exclude larger molecules. Further, membranes or hydrogels which allow passage of molecules up to about the size of immunoglobulin M (about 1,000 kD) can be used; only very large substances, such as cells, will be excluded in this embodiment.

The jacket is also biocompatible. That is, it does not elicit a detrimental host response sufficient to result in rejection of the implanted vehicle or to render it inoperable. Neither does the jacket elicit unfavorable tissue responses such as fibrosis. In addition, the external surface can be selected or designed in such a manner that it is particularly suitable for implantation at the selected site. For example, the external surface can be smooth, stippled or rough, depending on whether attachment by cells of the surrounding tissue is desirable. The shape or configuration can also be selected or designed to be particularly appropriate for the implantation site chosen.

The jacket of the present vehicle is further immunoisulatory. That is, it protects cells in the core of the vehicle from the immune system of the individual in whom the vehicle is implanted. It does so (1) by preventing harmful substances of the individual's body

-8-

from entering the core of the vehicle, (2) by minimizing contact between the individual and inflammatory, antigenic, or otherwise harmful materials which may be present in the core and (3) by providing a spatial barrier sufficient to prevent immunological contact between the isolated cells and detrimental portions of the individual's immune system. The vehicle of the present invention is distinguished from the microcapsules of Lim and Sun (Lim, F., Sun, A.M., Science 210:908-910 [1980]; Sun, A.M., Methods in Enzymology 137: 575-579 [1988]) by its outer jacket which ensures that cells cannot project outside of the core. The capsules of Lim and Sun suffered from the disadvantage that portions of encapsulated cells could potentially project from the core through the poly-L-lysine layer and thereby be more likely to elicit inflammatory responses from the host's immune system. This microcapsule technology relies on the presence of potentially bioactive ionic bonds to form the microcapsule. By virtue of their ionic nature, these microcapsules are susceptible to deterioration following implantation due to competition for the ionic bonds that take place in the body of the host after capsule implantation. This problem is minimized by the relatively non-ionic macrocapsules of the present invention. A further advantage of the macrocapsules of the present invention lies in their capacity to contain more cells in a single device than is possible in microcapsule technology.

The surrounding or peripheral region (jacket) can be made of a hydrogel matrix or of a different material, such as a thermoplastic membrane. It can also be made of a matrix-membrane composite, such that a permselective thermoplastic membrane having matrix-filled pores, is formed.

-9-

Suitably, the external jacket may be formed of a thermoplastic material known to be biocompatible, such as the ones described herein. In addition, other jackets which have been used in the microcapsule field may also be used herein, such as alginate, suitably cross-linked with a multivalent ion such as calcium.

The immunoisulatory vehicle is useful (a) to deliver a wide range of cellular products, including high molecular weight products, to an individual in need of them, and/or (b) to provide needed metabolic functions to an individual, such as the removal of harmful substances. The instant vehicle contains a multiplicity of cells, such that implantation of a few or a single vehicle is sufficient to provide an effective amount of the needed substance or function to an individual. A further advantage offered by the instant vehicle is practicality of retrieval.

In one embodiment of the preferred invention, which is particularly useful with islets of Langerhans, both the core and the surrounding or peripheral region of the instant vehicle are hydrogels, which can be the same composition hydrogel or different composition hydrogels.

This invention relates further to a method of making a biocompatible immunoisulatory vehicle. In a first embodiment, the vehicle is formed by coextruding from a nested-bore extrusion nozzle materials which form the core and surrounding or peripheral regions, under conditions sufficient to gel, harden, or cast the matrix or membrane precursor(s) of the surrounding or peripheral region (and of the core region). A particular advantage of this coextrusion embodiment is that the cells in the core are isolated from the moment of formation of the vehicle, ensuring that the core materials do not become contaminated or adulterated during handling of the vehicle

-10-

prior to implantation. A further advantage of the coextrusion process is that it ensures that the surrounding or peripheral region is free of cells and other core materials. The permeability and  
5 biocompatibility characteristics of the surrounding or peripheral region are determined by both the matrix or membrane precursor materials used, and the conditions under which the matrix or membrane is formed.

In a second embodiment of the present method, the  
10 immunoisulatory vehicle is formed stepwise. For example, if the immunoisulatory vehicle being made includes a hydrogel core containing the isolated cells, the core can be formed initially, and the surrounding or peripheral matrix or membrane can be assembled or applied  
15 subsequently. Conversely, the surrounding or peripheral matrix or membrane can be preformed, and then filled with the preformed isolated-cell containing core material or with materials which will form the core (i.e., core precursor materials). The vehicle is sealed in such a  
20 manner that the core materials are completely enclosed. If a core precursor material is used, the vehicle is then exposed to conditions which result in formation of the core.

This invention relates also to a method of delivering a  
25 biological product or altering a metabolic or immunologic function in an individual in need of the biological product or altered metabolic function. An immunoisulatory vehicle of the present invention is implanted into the individual (referred to as the recipient), using known  
30 techniques or methods and selected for the particular immunoisulatory vehicle and site of implantation. Once implanted, cells isolated within the biocompatible immunoisulatory vehicle produce the desired product(s) or perform the desired function(s). If products are released  
35 by the isolated cells, they pass through the surrounding



-11-

or peripheral permselective membrane or hydrogel matrix into the recipient's body. If metabolic functions are provided by the isolated cells, the substances to be metabolized (e.g., degraded or inactivated) enter the vehicle from the recipient's body and are removed from the recipient's bloodstream.

Thus, this invention further relates to a method of isolating cells within a biocompatible, immunoisulatory implantable vehicle, thereby protecting the cells within the vehicle from immunological attack after being implanted into an individual. Although some low molecular weight mediators of the immune responses (e.g. cytokines) may be permeable to the membrane, in most cases local or circulating levels of these substances are not high enough to have detrimental effects. The isolated cells are protected from attack by the recipient's immune system and from potentially deleterious inflammatory responses from the tissues which surround the implanted vehicle. In the core of the vehicle, the isolated cells are maintained in a suitable local environment. In this manner, needed substances or metabolic functions can be delivered to the recipient even for extended periods of time, and without the need to treat the recipient with dangerous immunosuppressive drugs.

#### 25 Brief Description of the Figures

Figure 1 is a graphic representation of the differences in the permeability of alginate matrices formed under different conditions to test solutes of various molecular sizes.

30 Figure 2 is a graphic representation of the results of a perfusion test of the functionality of immunoisolated versus unprotected islets maintained in vitro for four weeks. Figure 2A depicts the results obtained with an immunoisulatory vehicle having a hydrogel core matrix and  
35 a peripheral jacket made of a permselective thermoplastic

-12-

membrane. Figure 2B depicts the results obtained with an immunoisulatory vehicle having a hydrogel core matrix and a peripheral hydrogel jacket.

Figure 3 is a graphic representation showing the total amount of insulin released, and the amounts released during the first and second phase responses in the perifusion test also shown in Figure 2. Figure 3A depicts the results obtained with the dual-matrix immunoisulatory vehicle and Figure 3B depicts the results obtained with the core matrix-permselective membrane immunoisulatory vehicle.

Figure 4 is a graphic representation of the decrease in plasma glucose levels observed when immunoisolated xenogeneic islets are implanted into streptozotocin-induced diabetic mice for a period of 60 days. The immunoisulatory vehicle used was of the configuration described in Example 5.

Figure 5 is a graphic representation of insulin release in a perifusion experiment using an immunoisulatory vehicle containing rat islets, of the configuration described in Example 5, recovered after a period of residence in vivo and challenged with glucose, with and without theophylline stimulation.

Figure 6 is a graphic representation of the decrease in plasma glucose levels observed when immunoisolated xenogeneic islets are implanted into streptozotocin-induced diabetic mice for a period of 100 days. The immunoisulatory vehicle used was of the configuration described in Example 4.

Figure 7 is a graphic representation of the permeability of an alginate matrix to various test solutes. Permeabilities were tested after storage in Hank's

-13-

solution after 16 hours and 160 hours. The change in permeability is due to leaching of  $\text{Ca}^{++}$  from the matrix.

Figure 8 is a graphic comparison of the response to glucose challenge of rat islets isolated within dual matrix immunoisulatory vehicles with either thermoplastic or alginate jackets, following a period of residence in vivo in discordant xenogeneic recipients (guinea pigs).

Figure 9 is a graphic representation of the partial restoration of normal motor behavior to rodents with experimentally induced Parkinson-like behavior, following implantation of an immunoisulatory vehicle containing adrenal chromaffin cells in a core matrix, with a surrounding jacket of permselective thermoplastic membrane.

Figure 10 is a graphic representation of the mean body weight changes seen in quinolinic acid lesioned rats. Rats receiving immunoisulatory capsules containing bovine adrenal chromaffin cells maintained body weight significantly better than the other lesioned rats.

Figure 11 is a graphic representation of the nonfasting plasma glucose concentrations of diabetic mice after implantation of type 2 acrylic copolymer hollow fibers containing either 1000 rat islets (A) or 500 rat islets (B) implanted either intraperitoneally (circles) or subcutaneously (squares).

Figure 12 is a graphic representation of the effects on blood glucose in diabetic rats implanted with rat islets encapsulated in flat sheet devices.

#### Detailed Description of the Invention

This invention relates to a biocompatible immunoisulatory vehicle suitable for long-term implantation into

-14-

individuals. Because of its biocompatibility, the vehicle is suitable for long-term isolation of biologically useful cells and/or substances from the various protective systems of the body. As used herein, the term "protective systems" refers to the types of immunological attack which can be mounted by the immune system of an individual in whom the instant vehicle is implanted, and to other rejection mechanisms, such as the fibrotic response, foreign body response and other types of inflammatory response which can be induced by the presence of a foreign object in the individuals' body. Implants of the vehicle and contents thereof as described by the current invention retain functionality for greater than three months *in vivo* and in many cases for longer than a year. In addition, the vehicle of the current invention may be prepared of sufficient size to deliver an entire therapeutic dose of a substance from a single or just a few (less than 10) implanted and easily retrievable vehicles.

As used herein, the term "biocompatible" refers collectively to both the intact vehicle and its contents. Specifically, it refers to the capability of the implanted intact vehicle and its contents to avoid detrimental effects of the body's various protective systems and remain functional for a significant period of time. In addition to the avoidance of protective responses from the immune system, or foreign body fibrotic response, "biocompatible" also implies that no specific undesirable cytotoxic or systemic effects are caused by the vehicle and its contents such as would interfere with the desired functioning of the vehicle or its contents.

Important for biocompatibility and continued functionality are vehicle morphology, hydrophobicity and the absence of undesirable substances either on the surface of, or leachable from, the vehicle itself. Thus, brush surfaces, folds, interlayers or other shapes or structures eliciting

-15-

a foreign body response are avoided. The vehicle-forming materials are sufficiently pure that unwanted substances do not leach out from the vehicle materials themselves. Additionally, following vehicle preparation, the treatment of the external surface of the vehicle with fluids or materials (e.g. serum) which may adhere to or be absorbed by the vehicle and subsequently impair vehicle biocompatibility are avoided.

This invention also pertains to a method of isolating or protecting implanted cells, tissues, or other materials from immunological attack. The method and vehicle of the instant invention are useful to deliver a wide range of cellular products, including high molecular weight products, to an individual in need of them, and/or to provide needed metabolic functions to an individual, such as the removal of harmful substances. Products which can be delivered using the instant vehicle include a wide variety of factors normally secreted by various organs or tissues. For example, insulin can be delivered to a diabetic patient, dopamine to a patient suffering from Parkinson's disease, or Factor VIII to a Type A hemophiliac. Other products which can be delivered through use of the instant vehicle include trophic factors such as erythropoietin, growth hormone, Substance P, and neurotensin. Another family of products suited to delivery by the instant vehicle comprises biological response modifiers, including lymphokines and cytokines. Antibodies from antibody secreting cells may also be delivered. Specific antibodies which may be useful include those towards tumor specific antigens. The release of antibodies may also be useful in decreasing circulating levels of compounds such as hormones or growth factors. These products are useful in the treatment of a wide variety of diseases, inflammatory conditions or disorders, and cancers. The instant vehicle can also be used to restore or augment vital metabolic functions, such

-16-

as the removal of toxins or harmful metabolites (e.g., cholesterol) from the bloodstream by cells such as hepatocytes. The method and vehicle of the instant invention make possible the implantation of cells without  
5 the concomitant need to immunosuppress the recipient for the duration of treatment. Through use of the biocompatible immunoisulatory vehicle, homeostasis of particular substances can be restored and maintained for extended periods of time. The instant vehicle may contain  
10 a multiplicity of cells, such that implantation of a single vehicle can be sufficient to provide an effective amount of the needed substance or function to an individual.

The biocompatible immunoisulatory vehicle of the instant  
15 invention comprises (a) a core which contains a biologically active moiety, either suspended in a liquid medium or immobilized within a hydrogel or extracellular matrix, and (b) a surrounding or peripheral region of permselective matrix or membrane (jacket) which does not  
20 contain isolated cells, which is biocompatible, and which is sufficient to protect isolated cells if present in the core from immunological attack.

For purposes of the present invention, a biologically active moiety is a tissue, cell, or other substance, which  
25 is capable of exerting a biologically useful effect upon the body of an individual in whom a vehicle of the present invention containing a biologically active moiety is implanted. Thus, the term "biologically active moiety" encompasses cells or tissues which secrete or release a  
30 biologically active solute; cells or tissues which provide a metabolic capability or function, such as the removal of specific solutes from the bloodstream; or a biologically active substance such as an enzyme, trophic factor, hormone, or biological response modifier.

-17-

When the biologically active moiety within the core of the biocompatible immunoisulatory vehicle comprises cells, the core is constructed to provide a suitable local environment for the continued viability and function of the cells isolated therein. The instant vehicle can be used to immunoisolate a wide variety of cells or tissues, spanning the range from fully-differentiated, anchorage-dependent cells or primary tissues, through incompletely-differentiated fetal or neonatal tissues, to anchorage-independent transformed cells or cell lines.

Many transformed cells or cell lines are most advantageously isolated within a vehicle having a liquid core. For example, PC12 cells (which secrete dopamine and are herein shown to be useful for the treatment of Parkinsonism) can be isolated within a vehicle whose core comprises a nutrient medium, optionally containing a liquid source of additional factors to sustain cell viability and function, such as fetal bovine or equine serum.

Suitably, the core may be composed of a matrix formed by a hydrogel which stabilizes the position of the cells in cell clumps. The term "hydrogel" herein refers to a three dimensional network of cross-linked hydrophilic polymers. The network is in the form of a gel substantially composed of water, preferably but not limited to gels being greater than 90% water. Cross-linked hydrogels can also be considered solids because they do not flow or deform without appreciable applied shear stress.

Compositions which form hydrogels fall into two classes for the purposes of this application. The first class carries a net negative charge and is typified by alginate. The second class carries a net positive charge and is typified by extracellular matrix components such as collagen and laminin. Examples of commercially available

-18-

extracellular matrix components include Matrigel™ and Vitrogen™.

By way of example, cells which do not require an anchorage substrate are those which are able to form clumps or  
5 agglomerates and thus provide anchorage for each other. Exemplary clumping cell types are pancreatic islets, pancreatic beta-cell lines, Chinese hamster ovary (CHO) cells, and adrenal chromaffin cells. These cells are suitably enclosed in a negatively charged matrix such as  
10 alginate.

Fibroblasts generally survive well in a positively charged matrix and are thus suitably enclosed in extracellular-matrix type hydrogels. Certain cell types tend to multiply rapidly and could overgrow the space available  
15 within the core if they do not exhibit growth arrest. If the isolated cells do not exhibit growth arrest upon confluence, substances which induce quiescence can be included in the interior of the vehicle. In some instances, a hydrogel core may suffice to limit continued  
20 proliferation. For example, a hydrogel matrix precursor solution can be included but not exposed to polymerizing conditions. In the case of sodium alginate, a hydrogel will form slowly after implantation as calcium ions are scavenged from the surrounding tissues. Alternatively,  
25 growth inhibitory factors, or stimulators of differentiation can be incorporated into microbeads of a slowly degraded polymer such as polycarbonate, and cosuspended with the product-secreting cells. For instance, NGF or FGF can be used to stimulate PC-12 cell  
30 differentiation and terminate cell division.

Other cells, particularly primary cells or tissues, tend to adhere to each other and form dense agglomerations which develop central necrotic regions due to the relative inaccessibility of nutrients and oxygen to cells embedded



-19-

within the agglomerated masses. These dense cellular masses can form slowly, as a result of cell growth into dense colonies, or rapidly, upon the reassociation of freshly-dispersed cells or tissue mediated by cell-surface adhesion proteins. Cells or tissues which are highly metabolically active are particularly susceptible to the effects of oxygen or nutrient deprivation, and die shortly after becoming embedded in the center of an agglomerate. Many endocrine tissues, which normally are sustained by dense capillary beds, exhibit this behavior; islets of Langerhans and adrenal chromaffin cells are particularly sensitive. Cells or tissues which exhibit this behavior perform most satisfactorily in vehicles comprising a hydrogel matrix core, sufficient to immobilize the cells or tissue, thereby preserving the access of nutrients and oxygen to the majority of them. In other circumstances, the immobilizing hydrogel matrix further performs the additional function of producing or preserving functional units of a size and/or shape appropriate for maintaining desirable characteristics of the isolated cells. Moreover, the presence of the core matrix allows maintenance of a uniform distribution of cells or clusters of cells within the vehicle (i.e., the core matrix prevents settling and decreases mobility of the included cells).

One particularly advantageous use of hydrogel cores pertains to the encapsulation of actively dividing cells. Alginate or other hydrogels may be included in suspensions of actively dividing cells to be encapsulated. Following encapsulation and generation of the gel, the encapsulated cells are somewhat immobilized within the gel and new cells produced during cell division stay localized near the parent cell. In this manner clusters of cells are produced within the core. Such a growth method is advantageous in the case of cells such as the beta cell derived NIT cell line. In the absence of a core matrix

-20-

these cells tend to grow attached along the inner walls of the encapsulation device, with very few cells growing freely within the cavity of the device. Growth only on the walls of the capsule leads to a cell population size that is restricted by the surface area of the inner capsule wall as opposed to a population that grows to fill the vehicle cavity. When alginate is included in the core, cell growth is no longer limited to the inner capsule surface. Rather, discrete spheres of NIT cells are produced throughout the core, resulting in a significantly larger total cell population than that which occurs in the absence of alginate.

Even in the presence of a core matrix, the size of tissue fragments which can be loaded into a given vehicle volume is limited by the appearance of central necrosis within the individual fragments. In one aspect of the instant invention, the useful amount of tissue fragments or cell clusters that can be placed within the immunoisulatory vehicle is increased by preparing the cells in a form with improved diffusional characteristics. Generally this means preparation of the tissue fragments to a size less than 75 $\mu$ m diameter and most optimally to about 35 $\mu$ m diameter or less for vehicles to be implanted peritoneally. Aggregates or clusters of cells in improved diffusional form are prepared for spontaneously reaggregating cells (e.g. pancreatic islets or adrenal chromaffin cells) by enzymatically dispersing the tissue, to single cell and small cell aggregate suspensions, followed by controlled reaggregation to the improved diffusional form.

Herein, the term "aggregating" refers to a process of promoting cell clustering. The cells which form clusters may be obtained from naturally occurring agglomerates, such as pancreatic islets which are dispersed into single or small-clump suspension and subsequently reaggregated

-21-

according to the methods described. Alternatively, the cells may be obtained originally as single cells or small cell clumps, and then aggregated to form the desired cluster size. Such cell clusters will generally contain about 3 - 400 cells, depending upon cell size and aggregation characteristics. Typically, the cluster contains about 10 to about 50 cells. The use of reaggregated pancreatic islet cells is advantageous for insuring proper diffusion characteristics within the core and maintaining islet viability. Reaggregated islets also allow the use of smaller capsules. For instance, 500 non-reaggregated islets would generally require a capsule of approximately 14 cm in length (2% density). In contrast, capsules containing islets reaggregated to a size smaller than an intact islet may be as small as 1 to 2 cm in length because of more efficient packing. More efficient packing allows a lower  $pO_2$  outside the fiber to be tolerated without resultant necrotic cores. Built in tolerance for lower outside  $pO_2$  has at least two advantages. Firstly, a smaller capsule size may be used to contain the same number of cells, i.e. increased tissue density within the implant is better tolerated. Secondly, implantation sites with known reduced  $pO_2$ , such as subcutaneous locations, may be used successfully. The presence of the alginate matrix further insures that the aggregates will not reassociate to large cell masses where internal cells would be deprived of nutrients and/or oxygen.

Pancreatic islet cells still retain functionality and secrete insulin in response to glucose in near normal fashion following enzymatic dispersion and reaggregation. Cells from dispersed islets are reaggregated to the desired cluster size prior to loading into the vehicle. Reaggregation can be accomplished by the methods described by Britt, Diabetes 34: 898-903, or by similar methods. The optimal aggregate size for islets is the smallest size

-22-

- cluster which still maintains the desired physiological characteristics. The matrix or matrix forming materials may then be added to the cells, and the combination may be coextruded into or loaded into biocompatible
- 5 immunoisulatory vehicles. If necessary, matrix formation may then be induced. In preferred embodiments, cells are reaggregated overnight at 37°C, without agitation. The development of aggregates is monitored by light microscopy until aggregates achieve a size of 25 to 75µm preferably
- 10 35µm diameter. Liquid uncrosslinked alginate is then added to the cells to a concentration of from 0.5 to 2%, the cells are incorporated into vehicles, the vehicles are sealed if necessary, and polymerization is induced by immersion of the vehicle in CaCl<sub>2</sub>.
- 15 Primary cells or tissues may be useful with the vehicle of the instant invention for various medical applications. For regulatory reasons and reasons of patient safety, it may sometimes be useful to employ as sources for primary cultures, animals of carefully controlled hereditary and
- 20 developmental background. The presence of unwanted virus, bacteria and other pathogens may be limited through the use of specific pathogen free or gnotobiotic source animals. References and methods for the establishment, care and use of specific pathogen free and gnotobiotic
- 25 herds are provided by Maniats, O.P., et al. (1978) Can. J. Med. 42:428, Matthews P.J., et al., (1981) Recent Advances in Germ Free Research 61-64, Tokai Univ. Press, and in the National Accreditation Standards publication of the National SPF Swine Accrediting Agency, Inc., Conrad, Iowa.
- 30 Optionally, a matrix core can also contain materials which support or promote the function of the isolated cells. For example, extracellular matrix (ECM) components can be included to promote specific attachment or adhesion of the isolated cells. A combination of ECM components which is
- 35 particularly suitable for fostering the growth of certain

-23-

types of cells is taught in Kleinman et al., (1989) U.S. Patent No. 4,829,000. The core matrix can provide a reservoir for soluble or releasable substances, such as growth factors or growth regulatory substances, or for  
5 natural or synthetic substances which enhance or improve the supply of nutrients or oxygen to the isolated cells. Thus, it can function in a manner similar to the bone marrow ECM, which has been reported to behave as a slow-release reservoir for myeloid lineage-specific growth  
10 factors such as granulocyte-macrophage colony stimulating factor (gmcsf). Gordon, M.Y., et al., (1987) Nature 326:403-405. Thus, the core matrix can function as a reservoir for growth factors (e.g., prolactin, or insulin-like growth factor 2), growth regulatory substances such  
15 as transforming growth factor  $\beta$  (TGF $\beta$ ) or the retinoblastoma gene protein or nutrient-transport enhancers (e.g., perfluorocarbons, which can enhance the concentration of dissolved oxygen in the core). Certain of these substances are also appropriate for inclusion in  
20 liquid media.

Additionally, a population of feeder or accessory cells can be coisolated within the vehicle. For example, hepatocytes can be coisolated with endothelial accessory cells, Cai, Z., et al., (1988) Artificial Organs,  
25 12(5):388-393, or mixed with islet cells, Ricordi C., et al., (1987) Transplantation 45(6):1148-1151, or adrenal chromaffin cells can be coisolated with accessory cells which provide nerve growth factor (NGF), a substance needed by the chromaffin cells. In the latter case,  
30 fibroblasts which have been transfected with an expression vector for NGF can be used as accessory cells.

The instant vehicle can also be used as a reservoir for the controlled delivery of needed drugs or biotherapeutics. In such cases, the core, rather than  
35 containing cells or tissues, contains a high concentration

-24-

of the selected drug or biotherapeutic. In addition, satellite vehicles containing substances which prepare or create a hospitable environment in the area of the body in which a biocompatible immunoisulatory vehicle containing isolated cells is implanted can also be implanted into a recipient. In such instances, the vehicle containing immunoisolated cells is implanted in the region along with satellite vehicles releasing controlled amounts of, for example, a substance which down-modulates or inhibits an inflammatory response from the recipient (e.g., anti-inflammatory steroids), or a substance which stimulates the ingrowth of capillary beds (i.e., an angiogenic factor).

The surrounding or peripheral region (jacket) of the instant vehicle is permselective, biocompatible, and immunoisulatory. It is produced in such a manner that it is free of isolated cells, and completely surrounds (i.e., isolates) the core, thereby preventing contact between any cells in the core and the recipient's body.

Turning first to the permselective nature of the jacket, it is formed in such a manner that it has a MWCO range appropriate both to the type and extent of immunological reaction it is anticipated will be encountered after the vehicle is implanted and to the molecular size of the largest substance whose passage into and out of the vehicle is desirable. The type and extent of immunological attacks which may be mounted by the recipient following implantation of the vehicle depend in part upon the type(s) of moiety isolated within it and in part upon the identity of the recipient (i.e., how closely the recipient is genetically related to the source of the biologically active moiety). When the implanted tissue is allogeneic to the recipient, immunological rejection may proceed largely through cell-mediated attack by the recipient's immune cells against the implanted cells.

-25-

When the tissue is xenogeneic to the recipient, molecular attack through assembly of the recipient's cytolytic complement attack complex may predominate, as well as the antibody interaction with complement.

- 5 The MWCO of the surrounding region must therefore be sufficiently low to prevent access of the substances required to carry out these attacks to the core, yet sufficiently high to allow delivery of the needed product to the recipient's body. It will therefore be apparent  
10 that the MWCO need not be strictly restricted to a range which excludes immunoglobulin G from the core. In fact, there are many cases in which higher MWCOs are not only permissible, but also advantageous. Indeed, higher MWCOs allow the delivery of a wide variety of useful products  
15 from immunoisolated cells, as well as the use of such cells to provide metabolic control of high molecular weight substances.

Thus, in appropriate cases, the peripheral or surrounding region can be made of materials which form permselective  
20 membranes or hydrogel matrices allowing the passage of molecules up to about the size of C1q (about 400 kD), the largest protein required for the assembly of the complement attack complex. Therefore, any cellular product or metabolite below about the size of C1q can pass  
25 freely through the vehicle. In other cases, it may still be desirable to exclude immunoglobulins. In such cases, materials which form matrices or membranes through which molecules which are equivalent to or larger than the size of immunoglobulin G (about 150 kD) cannot pass can be  
30 used. Cellular products or metabolites which are smaller than about 150 kD will still pass through the vehicle. In still other cases, where the patient is immunosuppressed or where the implanted tissue is syngeneic to the patient, a vigorous immunological attack is not likely to be  
35 encountered, and passage of a high molecular weight

-26-

molecule may be desired. In these latter cases, materials which allow passage of all molecules up to about the size of immunoglobulin M (about 1,000 kD) can be used. These materials will impede the passage of only very large  
5 substances, such as cells.

In another aspect of the invention, it has been found that a molecular weight cutoff for the jacket considerably higher than that previously contemplated may be employed while maintaining the viability and function of the  
10 encapsulated cells.

This permits the macrocapsules to be used in applications where the cells secrete a substance of high molecular weight. For this purpose, a macrocapsule with molecular cutoffs in excess of say 80 to 100 kD to as high as 200 to  
15 1000 or 2000 kD or more may be employed in accordance with the present invention.

Turning next to the biocompatibility of the surrounding or peripheral region (jacket), this property is produced by a combination of factors. First, the materials used to form  
20 the vehicle are substances selected based upon their ability to be compatible with, and accepted by, the tissues of the recipient of the implanted vehicle. Substances are used which are not harmful to the recipient or to the isolated biologically active moiety. Preferred  
25 substances include reversibly and irreversibly gellable substances (e.g., those which form hydrogels), and water-insoluble thermoplastic polymers. Particularly preferred thermoplastic polymer substances are those which are modestly hydrophobic, i.e. those having a solubility  
30 parameter as defined in Brandrup J., et al. (1989) Polymer Handbook 3rd Ed., John Wiley & Sons, NY, between 8 and 15, or more preferably, between 9 and 14 (Joules/m<sup>3</sup>)<sup>1/2</sup>. The polymer substances are chosen to have a solubility parameter low enough so that they are soluble in organic



-27-

solvents and still high enough so that they will partition to form a proper membrane. Such polymer substances should be free of labile nucleophilic moieties and be highly resistant to oxidants and enzymes even in the absence of stabilizing agents. The period of residence *in vivo* which is contemplated for the particular immunoisulatory vehicle must also be considered: substances must be chosen which are adequately stable when exposed to physiological conditions and stresses. There are many hydrogels and thermoplastics which are sufficiently stable, even for extended periods of residence *in vivo*, such as periods in excess of one or two years. Examples of stable materials include alginate (hydrogel) and PAN/PVC (thermoplastic).

Second, substances used in preparing the biocompatible immunoisulatory vehicle are either free of leachable pyrogenic or otherwise harmful, irritating, or immunogenic substances or are exhaustively purified to remove such harmful substances. Thereafter, and throughout the manufacture and maintenance of the vehicle prior to implantation, great care is taken to prevent the adulteration or contamination of the vehicle with substances which would adversely affect its biocompatibility.

Third, the exterior configuration of the vehicle, including its texture, is formed in such a manner that it provides an optimal interface with the tissues of the recipient after implantation. This parameter will be defined in part by the site of implantation. For example, if the vehicle will reside in the peritoneal cavity of the recipient, its surface should be smooth. However, if it will be embedded in the soft tissues of the recipient, its surface can be moderately rough or stippled. A determining factor will be whether it is desirable to allow cells of the recipient to attach to the external surface of the vehicle or if such attachment must be

-28-

avoided. An open-textured or sponge-like surface may promote the ingrowth of capillary beds, whereas a smooth surface may discourage excessive overgrowth by fibroblasts. Excessive overgrowth by fibroblasts is to be avoided, except where capillary undergrowth has occurred, as it may result in the deposition of a poorly-permeable basement membrane around the vehicle and walling off of the isolated cells from contact with the recipient's body.

Certain vehicle geometries have also been found to specifically elicit foreign body fibrotic responses and should be avoided. Thus vehicles should not contain structures having interlayers such as brush surfaces or folds. In general, opposing vehicle surfaces or edges either from the same or adjacent vehicles should be at least 1 mm apart, preferably greater than 2 mm and most preferably greater than 5 mm. Preferred embodiments include cylinders, "U"-shaped cylinders, and flat sheets or sandwiches.

The surrounding or peripheral region (jacket) of the biocompatible immunoisulatory vehicle can optionally include substances which decrease or deter local inflammatory response to the implanted vehicle, and/or generate or foster a suitable local environment for the implanted cells or tissues. For example antibodies to one or more mediators of the immune response could be included. Available potentially useful antibodies such as antibodies to the lymphokines tumor necrosis factor (TNF), and interferon (IFN ) can be included in the matrix precursor solution. Similarly, an anti-inflammatory steroid can be included. Christenson, L., et al., (1989) J. Biomed. Mat. Res. (23):705-718; Christenson, L., 1989, Ph.D. thesis, Brown University, incorporated by reference. Alternatively, a substance which stimulates angiogenesis (ingrowth of capillary beds) can be included; this may be particularly desirable where the isolated cells or tissues

-29-

require close contact with the recipient's bloodstream to function properly (e.g., insulin-producing islets of Langerhans). Cell which are genetically engineered to secrete antibodies may also be included in the matrix.

- 5 Turning now to the concept of immunoisolation, the surrounding or peripheral region further confers protection of the biologically active moiety from the immune system of the individual in whom the vehicle is implanted by preventing harmful substances of the
- 10 individual's body from entering the core of the vehicle, and by providing a physical barrier sufficient to prevent detrimental immunological contact between the isolated moiety and the individual's immune system. The thickness of this physical barrier can vary, but it will always be
- 15 sufficiently thick to prevent direct contact between the cells and/or substances on either side of the barrier. The thickness of this region generally ranges between 5 and 200 microns; thicknesses of 10 to 100 microns are preferred, and thickness of 20 to 50 microns are
- 20 particularly preferred. Types of immunological attack which can be prevented or minimized by the use of the instant vehicle include attack by macrophages, neutrophils, cellular immune responses (e.g. natural killer cells and antibody-dependent T cell-mediated
- 25 cytotoxicity [ADCC]), and humoral response (e.g. antibody-dependent complement mediated cytotoxicity).

As discussed above, the type and extent of immunological response by the recipient to the implanted vehicle will be influenced by the relationship of the recipient to the

30 isolated biologically active moiety. For example, if the isolated materials comprise syngeneic cells, these will not cause a vigorous immunological reaction, unless the recipient suffers from an autoimmunity with respect to the particular cell or tissue type within the vehicle. There

35 are several disease or deficiency states which have

-30-

recently been determined to have an autoimmune etiology, most notably Type I, insulin-dependent Diabetes mellitus, wherein the insulin secreting pancreatic islet  $\beta$  cells are destroyed by the individual's immune system. Fan, M.-Y.,  
5 et al., Diabetes, 39: 519-522 (1990).

Syngeneic cells or tissue are rarely available. In many cases, allogeneic or xenogeneic cells or tissue (i.e., from donors of the same species as, or from a different species than, the prospective recipient) may be available.  
10 The immunoisulatory vehicle allows the implantation of such cells or tissue, without a concomitant need to immunosuppress the recipient. Therefore, the instant vehicle makes it possible to treat many more individuals than can be treated by conventional transplantation  
15 techniques. For example, far more patients suffer from Type 1 diabetes than can be transplanted with human donor islets (in 1990, fewer than about 4,000 suitable cadaver organ donors became available in the U.S. for all organ transplants). The supply of donor porcine or bovine  
20 islets is far greater; if these xenoislets are appropriately immunoisolated according to the instant invention, the diabetic condition of a far greater number of patients can be remedied. The type and vigor of an immune response to xenografted tissue is expected to  
25 differ from the response encountered when syngeneic or allogeneic tissue is implanted into a recipient. This rejection may proceed primarily by cell-mediated, or by complement-mediated attack; the determining parameters in a particular case may be poorly understood. However, as  
30 noted previously, the exclusion of IgG from the core of the vehicle is not the touchstone of immunoprotection, because IgG alone is insufficient to produce cytolysis of the target cells or tissues. Using the macrocapsules of the present invention, preferably with allogeneic tissue,  
35 but even with xenografts, it is possible to deliver needed high molecular weight products or to provide metabolic

-31-

functions pertaining to high molecular weight substances, provided that critical substances necessary to the mediation of immunological attack are excluded from the immunoisulatory vehicle. These substances may comprise  
5 the complement attack complex component C1q, or they may comprise phagocytic or cytotoxic cells; the instant immunoisulatory vehicle provides a protective barrier between these harmful substances and the isolated cells. Thus, the present immunoisulatory vehicle can be used for  
10 the delivery even from allogeneic or xenogeneic cells or tissue, products having a wide range of molecular sizes, such as insulin, parathyroid hormone, interleukin 3, erythropoietin, albumin, transferrin, and Factor VIII.

In another embodiment of this invention, methods are  
15 provided for the treatment of diseases caused by neural degeneration. Examples of human diseases which are thought to be associated with neural degeneration include Alzheimer's disease, Huntington's chorea, AIDS-related dementia, and Parkinson's disease.

20 Animal models for neurodegenerative conditions are based on the premise that a specific insult may damage or kill neurons. In some cases this may even lead to a cascade of neuronal death which affects trophically interdependent neurons along pathways responsible for specific brain  
25 functions.

A strategy for treatment of neural degenerative condition involves the localized administration of growth or trophic factors in order to (1) inhibit further damage to post-synaptic neurons, and (2) improve viability of cells  
30 subjected to the insult. Factors known to improve neuronal viability include basic fibroblast growth factor, ciliary neurotrophic factor, brain-derived neurotrophic factor, neurotrophin-3, neurotensin, and Substance P.

-32-

In one animal model for neurodegenerative excitotoxicity, the glutamate analog, quinolinic acid, is injected stereotaxically into the brain region known as the striatum and/or basal ganglia to produce neuropathology and symptoms analogous to those of patients suffering from Huntington's disease. Both the model and actual Huntington's disease are characterized by damage to neurons necessary for aspects of motor control.

Furthermore, one of the early symptoms of Huntington's disease is loss of body weight (Sanberg, P.R. et al. 1981 Med J Aust. 1, 407-409. Similar effects are also seen in the model system (Sanberg, P.R. et al. 1979 Exp Neurol 66, 444-466. Quinolinic acid is also found at abnormally high levels in AIDS-related dementia.

According to the present invention, trophic factors are provided to the proper brain region by implanting a vehicle containing living cells which secrete an appropriate factor. Suitably, the cells are adrenal chromaffin cells which are known to secrete at least one factor, basic fibroblast growth factor. Other as yet unidentified trophic factors may also be secreted by chromaffin cells. It is to be noted that this embodiment of the invention is separate from the use of chromaffin cells to secrete the neurotransmitter, dopamine, for the amelioration of symptoms of Parkinson's disease. Nerve growth factor-secreting cells such as fibroblasts engineered to express NGF represent an alternative therapy for this quinolinic acid induced neurodegeneration. Schwann cells prepared from myelin may be encapsulated and implanted in appropriate brain areas to prevent neural degeneration associated with Parkinson's disease

In another embodiment of the invention, the animal model involves lesion of the fimbria-fornix. In particular, neurons of the septohippocampal system are axotomized

-33-

which leads to degeneration and cell death. This model is thought to be analogous to the types of lesions which cause Alzheimer's disease in humans. Suitably, a growth factor, nerve growth factor (NGF), is provided by the

5 implantation of a vehicle containing cells which secrete NGF. Astrocytes immortalized (e.g. by transformation with the Large T antigen) and genetically engineered to express NGF may be employed. Preferably, the cells are fibroblasts which have been genetically engineered to

10 produce recombinant NGF. The fibroblasts survive best in a core composed of a matrix material which mimicks extracellular matrix, such as collagen or laminin-containing hydrogels. The core is surrounded by an immunoisulatory jacket which allows the diffusion of

15 oxygen and nutrients to the cells in the core, and also allows the secreted NGF to diffuse through the jacket and into the body of the recipient. The vehicle implant inhibits the death of cholinergic neurons as assayed by the number of neurons which contain choline acetyl

20 transferase (ChAT), an indicator of viable cholinergic neurons.

Fimbria-fornix lesions also cause behavioral deficits in the animal subjects of the model, most easily observed in

25 tasks involving learning and memory. It has been reported that chronic administration of NGF to rats with fimbria-fornix lesions accelerates the animals' behavioral recovery (Wills, B. et al. 1985 Behav. Brain Res. 17, 17-24). In the present invention, implantation of the

30 vehicle containing NGF-secreting cells provides a practical way to deliver NGF continuously to the appropriate brain region of the lesioned animal. By analogy, the vehicle of the present invention offers a practical form of regenerative and/or prophylactic therapy

35 for Alzheimer's victims whose conditions may be ameliorated by continuous delivery of NGF to specific brain regions.

-34-

The present immunoisulatory vehicle can be formed in a wide variety of shapes and combinations of suitable materials. A primary consideration in selecting a particular configuration for the vehicle when cells are present is the access of oxygen and nutrients to the isolated cells or tissues, and passage of waste metabolites, toxins and the secreted product from the vehicle. The immunoisulatory vehicle can be any configuration appropriate for maintaining biological activity and providing access for delivery of the product or function, including for example, cylindrical, rectangular, disk-shaped, patch-shaped, ovoid, stellate, or spherical. Moreover, the vehicle can be coiled or wrapped into a mesh-like or nested structure. If the vehicle is to be retrieved after it is implanted, configurations which tend to lead to migration of the vehicle(s) from the site of implantation, such as spherical vehicles small enough to travel in the recipient's blood vessels, are not preferred. Certain shapes, such as rectangles, patches, disks, cylinders, and flat sheets offer greater structural integrity and are preferable where retrieval is desired.

The instant vehicle must provide, in at least one dimension, sufficiently close proximity of any isolated cells in the core to the surrounding tissues of the recipient, including the recipient's bloodstream, in order to maintain the viability and function of the isolated cells. However, the diffusional limitations of the materials used to form the vehicle do not in all cases solely prescribe its configurational limits. Certain additives can be used which alter or enhance the diffusional properties, or nutrient or oxygen transport properties, of the basic vehicle. For example, the internal medium can be supplemented with oxygen-saturated perfluorocarbons, thus reducing the needs for immediate contact with blood-borne oxygen. This will allow isolated



-35-

cells or tissues to remain viable while, for instance, a gradient of angiotensin is released from the vehicle into the surrounding tissues, stimulating ingrowth of capillaries. References and methods for use of perfluorocarbons are given by Faithful, N.S. (1987) Anaesthesia 42: 234-242 and NASA Tech Briefs MSC-21480, U.S. Govt. Printing Office, Washington, D.C. 20402, incorporated herein by reference. Alternatively for clonal cell lines such as PC12 cells, genetically engineered hemoglobin sequences may be introduced into the cell lines to produce superior oxygen storage. Vol. 15 #1 page 54. NPO-17517 NASA Tech Briefs.

In general, in the absence of oxygen carrier additives, when the cells are present the vehicle will have a maximum depth-to-surface distance of no more than 2 mm in at least one dimension, with a maximum depth of 800 microns being preferred. One or several vehicles may be required to produce the desired effect in the recipient.

The thickness of the immunoisulatory vehicle jacket should be sufficient to prevent an immunoresponse by the patient to the presence of the vehicles. For that purpose, the vehicles preferably have a minimum thickness of 1 $\mu$ m or more free of the cells.

Additionally, reinforcing structural elements can be incorporated into the vehicle. These structural elements can be made in such a fashion that they are impermeable, and are appropriately configured to allow tethering or suturing of the vehicle to the tissues of the recipient. In certain circumstances, these elements can act to securely seal the surrounding or peripheral region (e.g., at the ends of a cylindrical vehicle, or at the edges of a disk-shaped vehicle), completing isolation of the core materials (e.g., a molded thermoplastic clip). For many configurations, it is desirable that these structural

-36-

elements should not occlude a significant area of the permselective surrounding or peripheral region.

In one preferred embodiment, the implantable immunoisulatory vehicle of the present invention is of a sufficient size and durability for complete retrieval after implantation. To be contrasted with such microcapsules, which have a typical maximum practical volume on the order of  $1\mu\text{l}$ , the preferred immunoisulatory vehicle of the present invention is termed "macrocapsule".

Such macrocapsules have a core of a preferable minimum volume of about 1 to  $10\mu\text{l}$  and depending upon use are easily fabricated to have a value in excess of  $100\mu\text{l}$ .

In terms of retrievability, microspheres are generally less practical than macro-capsules. In order for tissue encapsulated in microspheres to provide a therapeutic dose of insulin, for instance, the number of microspheres must be increased to such a large extent that significant retrievability becomes impossible. Additionally, an increase in the volume of tissue placed within a microsphere requires a corresponding increase in surface area. Within a sphere, because surface area scales with  $r^2$  where as volume scales with  $r^3$ , as the volume of encapsulated tissue volume increases, the required capsule size to provide sufficient surface area for nutrient diffusion to the encapsulated tissue quickly becomes impractical.

Macrocapsules in the shapes of cylinders or flat sheets do not have these limitations because volume increases more proportionately to surface area such that the diffusional transport of nutrients and products for increased amounts of tissue can be accommodated by increasing the surface area without unwieldy increases in total vehicle size. If, for example, about 10,000 islets are required per kg body weight to restore normoglycemia to a diabetic

-37-

patient, from 1,000 to 10,000 microcapsules must be transplanted per kg body weight (e.g. 1-10 islets per capsule). This number of microcapsules could not be easily retrieved, if retrieval were required. In contrast, the macrocapsules of the instant invention may easily hold greater than 1,000 islets to as high as 500,000 islets or more per vehicle. The preferred embodiment would require fewer than 5-10 vehicles per patient, making macrocapsules more easily retrieved than a large number of microcapsules.

The macrocapsules of the present invention are distinguished from microcapsules (Sun, A.M., supra; Rha, C-K., U.S. Patent 4,744,933) by the capacity of macrocapsules to contain over  $10^4$  cells and maintain them in viable condition. The droplet methods used in the making of microcapsules, in order to ensure cell viability, necessarily limit the number of cells per capsule to fewer than  $10^4$ .

The instant invention also relates to a method for making an immunoisulatory vehicle. As noted previously, the vehicle can provide for the implantation of diverse cell or tissue types, including fully-differentiated, anchorage-dependent, fetal or neonatal, or transformed, anchorage-independent cells or tissue. The cells to be immunoisolated are prepared either from a donor (i.e., primary cells or tissues, including adult, neonatal, and fetal cells or tissues) or from cells which replicate in vitro (i.e., immortalized cells or cell lines, including genetically modified cells). In all cases, a sufficient quantity of cells to produce effective levels of the needed product or to supply an effective level of the needed metabolic function is prepared, generally under sterile conditions, and maintained appropriately (e.g. in a balanced salt solution such as Hank's salts, or in a nutrient medium, such as Ham's F12) prior to isolation.

-38-

In another aspect of the invention, the macrocapsules are of a shape which tends to reduce the distance between the center of the macrocapsule and the nearest portion of the jacket for purposes of permitting easy access of nutrients from the patient into the cell or of entry of the patient's proteins into the cell to be acted upon by the cell to provide a metabolic function, such as interaction with cholesterol or the like. In that regard, a non-spherical shape is preferred, such as a long tube or flat plate, or the like. The optimum shape for this purpose may be calculated by known techniques as set forth herein.

Four important factors influencing the number of cells or amount of tissue to be placed within the core of the biocompatible immunoisulatory vehicle (i.e., loading density) of the instant invention are: (1) vehicle size and geometry; (2) mitotic activity within the vehicle; (3) viscosity requirements for core preparation and or loading; and (4) pre-implantation assay and qualification requirements.

With respect to the first of these factors, (capsule size and geometry), the diffusion of critical nutrients and metabolic requirements into the cells as well as diffusion of waste products away from the cell are critical to the continued viability of the cells. Since diffusional access to the contents of the vehicle is limited by vehicle surface area, surface to volume relationships of various shapes and size vehicles will be critical in determining how much viable tissue can be maintained within the vehicle. Among the metabolic requirements met by diffusion of substances into the vehicle is the requirement for oxygen. The oxygen requirements of the specific cells must be determined for the cell of choice. Methods and references for determination of oxygen metabolism are given in Wilson D.F. et al., (1988) J. Biol. Chem. 263:2712-2718. The oxygen requirement for

-39-

islet cells has been applied to coupled diffusion reaction models accounting for diffusional transport from surrounding tissue through the vehicle wall and tissue compartment (core), and used to calculate the expected viability of islet cells in a number of vehicles of different sizes and configurations, after the method of Dionne, K.E., (1989), Thesis Ph.D., Massachusetts Institute of Technology. For intact pancreatic islets, these calculations agree well with experimental observations. For a cylindrical vehicle of 900 microns outer diameter, implanted into the peritoneal cavity ( $pO_2 \approx 45-50$  mmHg), the optimal total cell volume is in the range of up to 20%, preferably 1 - 15%, most preferably about 5% of the vehicle volume. (If this capsule were 20cm in length it would have a volume of  $100\text{mm}^3$ . To provide the same amount of surface area with a single sphere, e.g. to support comparable amounts of tissue, would require a volume of  $1,047\text{mm}^3$ . For a cylindrical vehicle of 400 microns the optimal cell volume is between 35-65% total vehicle volume, and is preferably 35%. These calculations also take into account the partial oxygen pressure at the site of implantation. At implantation sites where the oxygen pressure is less than the peritoneum (e.g., subcutaneous  $pO_2 \approx 20$ ), lower loading densities will be required. Implantation into arteries ( $pO_2$  95mmHg) and the brain ( $pO_2 > 75$  mmHg) will allow support of greater tissue volume per unit vehicle. Other vehicle configurations, such as disk-shaped or spherical, are also possible and optimal cell volumes may be similarly calculated for these geometries. Actual loading densities will consider not only these diffusional considerations but also the other considerations given below.

With respect to the second factor (cell division), if the cells selected are expected to be actively dividing while in the vehicle, then they will continue to divide until they fill the available space, or until phenomena such as

-40-

contact inhibition limit further division. For replicating cells, the geometry and size of the vehicle will be chosen so that complete filling of the vehicle core will not lead to deprivation of critical nutrients due to diffusional limitations. In general, vehicles that will be filled to confluency with cells or tissue will be no more than 250 microns in cross-section, such that cells in the interior will have less than 15 cells between them and an external diffusional surface, preferably less than 10 cells and more preferably less than 5 cells.

In general, for cells not expected to divide within the vehicle, such as chromaffin cells, pancreatic islet cells and the like, the appropriate cell densities will be calculated from the diffusional considerations listed above.

With respect to the third factor (viscosity of core materials) cells in densities occupying up to 70% of the vehicle volume can be viable, but cell solutions in this concentration range would have considerable viscosity. Introduction of cells in a very viscous solution into the vehicle could be prohibitively difficult. In general, for both two step and coextrusion strategies, discussed below, cell loading densities of higher than 30% will seldom be useful, and in general optimal loading densities will be 20% and below. For fragments of tissues, it is important, in order to preserve the viability of interior cells, to observe the same general guidelines as above and tissue fragments should not exceed 250 microns in diameter with the interior cells having less than 15, preferably less than 10 cells between them and the nearest diffusional surface.

Finally, with respect to the fourth factor (preimplantation and assay requirements), in many cases, a certain amount of time will be required between vehicle

-41-

preparation and implantation. For instance, it may be important to qualify the vehicle in terms of its biological activity. Thus, in the case of mitotically active cells, preferred loading density will also consider  
5 the number of cells which must be present in order to perform the qualification assay.

In most cases, prior to implantation *in vivo* it will be important to use *in vitro* assays to establish the efficacy of the biologically active moiety within the vehicle.  
10 Vehicles containing the moiety of interest can be constructed and analyzed using model systems. In a preferred embodiment of the instant invention, the diffusion of glucose into the vehicle is used to stimulate insulin release from pancreatic islet cells. The  
15 appearance of insulin outside the vehicle is monitored through the use of an appropriately specific radioimmunoassay. Such procedures allow the determination of the efficacy of the vehicle on a per cell or unit volume basis.

20 Following the above guidelines for vehicle loading and for determination of vehicle efficacy, the actual vehicle size for implantation will then be determined by the amount of biological activity required for the particular application. In the case of secretory cells releasing  
25 therapeutic substances, standard dosage considerations and criteria known to the art will be used to determine the amount of secretory substance required. Factors to be considered include; the size and weight of the recipient; the productivity or functional level of the cells; and,  
30 where appropriate, the normal productivity or metabolic activity of the organ or tissue whose function is being replaced or augmented. It is also important to consider that a fraction of the cells may not survive the immunoisolation and implantation procedures, as well as  
35 whether the recipient has a preexisting condition which

-42-

can interfere with the efficacy of the implant. Vehicles of the instant invention can easily be manufactured which contain many thousands of cells. In preferred embodiments, therapeutically useful immunoisulatory vehicles used to provide insulin to insulin deficient rats contained on the order of 1,000 islets. Larger vehicles can also be conveniently prepared by the method of the current invention. Because of the potentially large capacity of the immunoisulatory vehicles, the treatment of many conditions will require only one or at most a few (less than 10) implanted vehicles to supply an appropriate therapeutic dose. The use of only a few therapeutically effective implantable vehicles containing a large number of cells provides simple retrievability which, for many applications, will be preferred over microsphere or other small configurations requiring a large number of vehicles. The immunoisulatory macrocapsule of the present invention is capable of storing 10,000 to 100,000 cells to as high as 500,000 cells or more, in individual or cluster form, depending on their type.

Techniques and procedures for isolating cells or tissues which produce a selected product are known to those skilled in the art, or can be adapted from known procedures with no more than routine experimentation. For example, islets of Langerhans can be isolated from a large-animal pancreas (e.g., human or porcine) using a combination of mechanical distention and collagenase digestion, as described by Scharp, D.W., et al., (1989) in U.S. Patent No. 4,868,121. Islets may be isolated from small animals such as rats by the method of Scharp, et al., (1980) Diabetes 29:suppl 1, 19-30. Similarly, hepatocytes can be isolated from liver tissue using collagenase digestion followed by tissue fractionation, as described by Sun, A.M., et al., (1987) Biomat., Art. Cells, Art. Org. 15(2):483-496. Adrenal Chromaffin cells may be isolated by the method of Livett, B.G. (1984)



-43-

Physiology Reviews 64:1103-1161. Many cellular products which are difficult to provide using primary donor tissues can be provided using immortalized cells or cell lines. Immortalized cells are those which are capable of

5 indefinite replication but which exhibit contact inhibition upon confluence and are not tumorigenic. An example of an immortalized cell line is the rat pheochromocytoma cell line PC12. Transformed cells or cell lines can be used in a similar manner. Transformed

10 cells are unlike merely immortalized cells in that they do not exhibit contact inhibition upon confluence, and form tumors when implanted into an allogeneic host. Immortalization can allow the use of rare or notoriously fragile cell or tissue types for the long-term delivery of

15 a chosen product or metabolic function. Suitable techniques for the immortalization of cells are described in Land H. et al. (1983) Nature 304:596-602 and Cepko, C.L., (1988) Neuron 1:345-353. Candidate cell lines include genetically engineered beta-cell lines which

20 secrete insulin such as NIT cells (Hamaguchi, K., et al., 1991 Diabetes 40:842), RIN cells (Chick, W.L., et al., PNAS 1977 74:628-632), ATT cells (Hughes, S.D., et al, 1992, PNAS USA 89:688-692), CHO cells (Matsumoto, M., et al, 1990, PNAS USA 87:9133-9137), and beta-TC-3 cells

25 (Tal, M., et al, 1992, Molec. and Cell Biol. 12:422-432). Additionally, recombinant cells or cell lines can be engineered to provide novel products or functions and combinations thereof, using a wide variety of techniques well known to those of ordinary skill in the art.

30 For example, fibroblasts can be transfected with an expression vector for the chosen product (e.g., nerve growth factor, erythropoietin, insulin, or Factor VIII). It should be recognized however, that expression of a recombinant protein in a cell type which does not normally

35 express the protein may lead to unregulated expression

-44-

which may not be desirable for certain medical applications.

B-cell hybridomas secreting a selected monoclonal antibody, or T-cell hybridomas secreting a selected lymphokine, can also be used. It may be particularly desirable to deliver a monoclonal antibody or fraction thereof, which neutralizes the biological activity of a disregulated biological response modifier using the instant invention. Engineered cells which secrete soluble fragments of receptors for these biological response modifiers can be used in a similar fashion. The disregulation or overproduction of particular biological response modifiers has been implicated in the etiology of certain cancers.

If the cells to be immunoisolated are replicating cells or cell lines adapted to growth in vitro, it is particularly advantageous to generate a cell bank of these cells. A particular advantage of a cell bank is that it is a source of cells prepared from the same culture or batch of cells. That is, all cells originated from the same source of cells and have been exposed to the same conditions and stresses. Therefore, the vials can be treated as identical clones. In the transplantation context, this greatly facilitates the production of identical or replacement immunoisulatory vehicles. It also allows simplified testing protocols which assure that implanted cells are free of retroviruses and the like. It may also allow for parallel monitoring of vehicles *in vivo* and *in vitro*, thus allowing investigation of effects or factors unique to residence *in vivo*.

In all cases, it is important that the cells or tissue contained in the vehicle are not contaminated or adulterated. If a vehicle having a matrix core is desired, the cells are mixed under sterile conditions,

-45-

with an appropriate amount of a biocompatible, gellable hydrogel matrix precursor. There are numerous natural and synthetic hydrogels which are suitable for use in a biocompatible immunoisulatory vehicle of the instant  
5 invention. Suitable naturally-derived hydrogels include plant-derived gums, such as the alkali metal alginates and agarose, and other plant-derived substances, such as cellulose and its derivatives (e.g., methylcellulose). Animal tissue-derived hydrogels such as gelatin are also  
10 useful. Alternatively, the core matrix can be made of extracellular matrix (ECM) components, as described by Kleinman et al., (1989) U.S. Patent No. 4,829,000. Suitable synthetic hydrogels include polyvinyl alcohol, block copolymer of ethylene-vinylalcohol, sodium  
15 polystyrene sulfonate, vinyl-methyl-tribenzyl ammonium chloride and polyphosphazene (Cohen, S. et al. 1990 J. Anal. Chem. Soc. 112:7832-7833).

If a dual matrix immunoisulatory vehicle is to be formed, the surrounding or peripheral region can be made of a  
20 hydrogel selected from the above-listed matrix precursors. If the surrounding or peripheral region of the vehicle is to comprise a permselective membrane, other precursor materials can be used. For example, the surrounding or peripheral region can be made from water-insoluble,  
25 biocompatible thermoplastic polymers or copolymers. Several of the polymers or copolymers taught by Michaels (1971) U.S. Patent No. 3,615,024, which is hereby incorporated by reference, fulfill these criteria. A preferred membrane casting solution comprises a  
30 polyacrylonitrile-polyvinylchloride (PAN/PVC) copolymer dissolved in the water-miscible solvent dimethylsulfoxide (DMSO). This casting solution can optionally comprise hydrophilic or hydrophobic additives which affect the permeability characteristics of the finished membrane. A  
35 preferred hydrophilic additive for the PAN/PVC copolymer is polyvinylpyrrolidone (PVP). Other suitable polymers

-46-

comprise polyacrylonitrile (PAN), polymethylmethacrylate (PMMA), polyvinylidene fluoride (PVDF), polyethylene oxide, polyolefins (e.g., polyisobutylene or polypropylene), polysulfones, and cellulose derivatives (e.g., cellulose acetate or cellulose butyrate). Compatible water-miscible solvents for these and other suitable polymers and copolymers are found in the teachings of U.S. Patent No. 3,615,024.

In a preferred embodiment, the core is surrounded by a biocompatible hydrogel matrix free of cells projecting externally from the outer layer. The macrocapsules of the present invention are distinguished from the microcapsules of Rha, Lim, and Sun (Rha, C.K. et al, U.S. Patent 4,744,933; Sun, A.W., supra) by (1) the complete exclusion of cells from the outer layer of the macrocapsule, and (2) the thickness of the outer layer of the macrocapsule. Both qualities contribute to the immunoisolation of encapsulated cells in the present invention. The microcapsules of Rha were formed by ionic interaction of an ionic core solution with an ionic polymer of opposite charge. The microcapsules of Lim and Sun were formed by linking an external hydrogel jacket to the core through an intermediate layer of poly-L-lysine (PLL).

In the microcapsules of Lim and Sun, the intermediate PLL layer was not sufficiently thick to guarantee that portions of the encapsulated cells would not penetrate through and beyond the layer. Cells penetrating the PLL layer are potential targets for an immune response. All these capsules, including those disclosed by Rha, also suffer the following additional limitations: (a) they are round, and (b) the formation of the outer layer is dependent upon direct ionic bonding or polyamide linkage with an inner layer or core substance. The disadvantages

-47-

of round shape and direct ionic bonding between polymers are described supra.

In the capsules of Rha, Lim, and Sun, since the chemical identity of the inner substance is either dictated by  
5 choice of outer layer, or PLL, the ability to vary growth conditions on the inside of these capsules is greatly limited. Since there are often specific growth conditions which need to be met in order to successfully encapsulate specific cell types, these capsules generally have a  
10 limited utility or require considerable experimentation to establish appropriate outer layers for a given internal substance. In contrast, in the instant invention, the identity of the core material does not place strict limitations on the identity of the outer jacket material  
15 or vice versa. This allows the material of the inner hydrogel to be selected according to criteria important for cell viability and growth, and the outer jacket material to be selected on the basis of immunoisulatory properties, biocompatibility, and/or manufacturing  
20 considerations.

The microcapsules of Rha, Lim, and Sun have a greater potential for bioincompatibility, fibrogenesis, and vehicle deterioration than do the macrocapsules of the present invention. A variety of biological systems are  
25 known to interact with and break down the ionic bonds required for the integrity of microcapsules. PLL evokes unfavorable tissue reactions to the capsule. Most notably, this is a fibrotic response. Thus, if there is any break in the external layer, if it is not of  
30 sufficient thickness, if the PLL layer begins to degrade, or if encapsulated cells are entrapped within the external layer sufficiently close to its outer surface, the microcapsule can trigger a fibrotic response. The term "fibrogenic" is used herein in reference to capsules or  
35 materials which elicit a fibrotic response in the

-48-

implantation site. As set forth herein, the external jacket of the immunoisulatory, non-fibrogenic macrocapsule of the present invention may be formed in a number of ways.

5 In one embodiment, the core is preformed by cross-linking a hydrogel matrix with a cross-linking agent, preferably a multivalent cation such as calcium. However, other known hydrogel cross-linking agents may also be employed. After cross-linking, the core is dipped into a solution of  
10 hydrogel to form a second layer free of cells in the core which, simultaneously or thereafter, is cross-linked suitably in the same manner. In the instant embodiment, cross-linking of the core material with the jacket material is accomplished via the cross-linking agent. For  
15 instance, when the core and jacket materials are both negatively charged hydrogels, the core and the jacket are cross-linked with each other via their mutual attraction to the positive charges on the cross-linking agent, preferably calcium. The core and jacket may be formed of  
20 the same or different type of hydrogel, provided that both have the same charge. Notably, the instant vehicle is not formed through direct ionic bonding between anionic and cationic polymers as described in U.S. patent 4,744,933 (Rha, CK, et al, May 17, 1988). Herein, the term "direct  
25 ionic bonding" refers to the type of chemical bonding in which two oppositely charged polymers are attracted to one another because of their oppositely charged moieties. The instant embodiment is distinguished from that of Rha because, in the instant embodiment, both the core material  
30 and jacket material have the same charge, and their association is via an oppositely charged cross-linking agent. This embodiment may be in the form of a microcapsule or a macrocapsule but, for reasons set forth herein, the macrocapsule form is preferred.

-49-

The instant vehicle can be formed either by coextrusion or stepwise assembly. Techniques for coextrusion which can be used to form the instant vehicle are taught in copending U.S. Patent Application Serial No. 07/461,999, filed January 8, 1990, which is herein incorporated by reference. For example, a coextrusion device similar to that taught by U.S.S.N. 07/461,999 is used in making the subject vehicle. The device has a nested-bore extrusion nozzle, the lumen of each bore (inner and outer) being appropriately connected to sterile chambers for delivery of the core and surrounding region materials. The nozzle can be of any configuration appropriate to produce an immunoisulatory vehicle whose shape is appropriate to the metabolic needs of the cells to be immobilized and the permeability and strength of the matrix which will surround them. For example, the nozzle can be circular, elliptical, stellate, or slot-shaped. Optionally, the nested bores can be coaxial. The widest aperture of the nozzle must be commensurate with the maximum diffusional depth appropriate to the vehicle being formed, including the metabolic needs of the isolated cells or tissues, and the materials of the core and peripheral regions. Upon extrusion of the core and peripheral region materials from the inner and outer chambers through the corresponding bores of the nozzle under conditions sufficient to gel, harden, or cast the matrix or membrane precursor(s) of the surrounding or peripheral region (and of the core region), an elongated vehicle of the selected shape is continuously formed. The length of the vehicle, and therefore its volume or capacity, can be controlled to produce vehicles of sizes appropriate for the particular use contemplated. An immunoisulatory vehicle formed by coextrusion in this manner is particularly preferred because use of this means ensures that the cells in the core are isolated from the moment of formation of the vehicle. Thus, it also ensures that the core materials do not become contaminated or adulterated during handling of the vehicle prior to

-50-

implantation. Furthermore, the nature of the coextrusion process is such that it ensures that the surrounding or peripheral region (jacket) is free of the materials in the core, including the cells, thus assuring that these cells will be immunoisolated when the vehicle is implanted into an individual. The permeability, molecular weight cutoff, and biocompatibility characteristics of the surrounding or peripheral region are determined by both the chosen matrix or membrane precursor materials used, and the conditions under which the matrix or membrane is formed.

The co-extruded vehicles may be formed with a hydrogel matrix core and a thermoplastic or hydrogel jacket. Such macrocapsules may be formed with a core and jacket of the same or different hydrogel which may be cross-linked to each other or not.

In reference to the permselectivity of the membranes and gels of the instant invention, the phrase "molecular weight cutoff" (MWCO) is used. It is recognized that there are many methods available for the determination of the molecular weight cutoff for a permselective membrane. Depending upon the method used, somewhat different MWCO estimates may be achieved for the same membrane. In the context of the current invention, MWCO refers to the results of the empirical determinations described herein, using the specific markers described under the specific conditions of the determination. Other methods of MWCO determination, to apply to the current invention, will need to be calibrated against the protocol of the instant invention according to methods known to practitioners in the art.

If a dual-matrix immunoisulatory vehicle (e.g., an alginate matrix) is formed, the permeability of the surrounding matrix can be determined by adjusting the concentration of matrix precursor used (e.g., sodium



-51-

alginate), and/or the concentration of gelling agent (in the case of alginate, a divalent cation such as  $\text{Ca}^{++}$ ) present in an immersion bath into which the materials are coextruded.

- 5 If an immunoisulatory vehicle with a surrounding or peripheral region of thermoplastic membrane is desired, the pore size range and distribution can be determined by varying the solids content of the solution of precursor material (the casting solution), the chemical composition
- 10 of the water-miscible solvent, or optionally including a hydrophilic or hydrophobic additive to the casting solution, as taught by U.S. Patent No. 3,615,024. The pore size may also be adjusted by varying the hydrophobicity of the coagulant and/or of the bath.
- 15 Typically, the casting solution will comprise a polar organic solvent containing a dissolved, water-insoluble polymer or copolymer. This polymer or copolymer precipitates upon contact with a solvent-miscible aqueous phase, forming a permselective membrane at the site of
- 20 interface. The size of pores in the membrane depends upon the rate of diffusion of the aqueous phase into the solvent phase; the hydrophilic or hydrophobic additives affect pore size by altering this rate of diffusion. As the aqueous phase diffuses farther into the solvent, the
- 25 remainder of the polymer or copolymer is precipitated to form a trabecular support which confers mechanical strength to the finished vehicle. The external surface of the vehicle is similarly determined by the conditions under which the dissolved polymer or copolymer is
- 30 precipitated (i.e., exposed to the air, which generates an open, trabecular or sponge-like outer skin, immersed in an aqueous precipitation bath, which results in a smooth permselective membrane bilayer, or exposed to air saturated with water vapor, which results in an
- 35 intermediate structure). Again, it will be readily appreciated that this method of forming the

-52-

immunoisulatory vehicle ensures that the peripheral or surrounding membrane is free of the cells in the core which are desired to be isolated from the immune system of the individual in whom the vehicle is to be implanted.

- 5 The surface texture of the vehicle is dependent in part on whether the extrusion nozzle is positioned above, or immersed in, the bath: if the nozzle is placed above the surface of the bath a roughened outer skin of PAN/PVC will be formed, whereas if the nozzle is immersed in the bath a  
10 smooth external surface is formed.

- The immunoisulatory vehicle can also be formed in a stepwise manner. For vehicles wherein the core comprises, in addition to the cells desired to be isolated, a hydrogel matrix, the core can be formed initially, and the  
15 surrounding or peripheral matrix or membrane can be assembled or applied subsequently. The matrix core can either be formed by extrusion or by molding. In a preferred embodiment, a patch- or sheet-shaped vehicle is formed by stepwise extrusion of calendered sheets. In  
20 this embodiment, a sheet of core material is layered onto a sheet of peripheral region material, then covered by a second sheet of peripheral region material. The edges of the vehicle are then sealed by crimping, compressing, heating, sealing with a biocompatible glue, or binding  
25 into a preformed biocompatible impermeable clip or combinations of the above.

- Conversely, the surrounding or peripheral matrix or membrane can be preformed, filled with the materials which will form the core (for instance, using a syringe), and  
30 subsequently sealed in such a manner that the core materials are completely enclosed. The vehicle can then be exposed to conditions which bring about the formation of a core matrix if a matrix precursor material is present in the core. Alternatively, a patch- or sheet-shaped

-53-

matrix core can be formed by molding, then sandwiched between sheets of permselective membrane and sealed or clipped in the manner described above to complete the isolation of the core materials.

5 It is also possible for a single, continuous hydrogel matrix to provide both immunoisolation and support or immobilization. For example, this can be accomplished by including the isolated cells within the vehicle distributed in a concentric gradient about the core, such  
10 that the peripheral region of the vehicle is free of the immobilized cells. Immunoisulatory vehicles of this nature can be made in at least two ways. First, a mixture of cells suspended in a solution of a hydrogel matrix precursor, which is denser than the isolated cells, can be  
15 extruded from the nozzle of a simple extrusion device. In this manner, the cells are forced into the core region of the forming vehicle. Alternatively, the cell-matrix precursor mixture can be extruded from the core lumen of a nested-bore nozzle, while simultaneously delivering a  
20 stream of a gelling agent (e.g., for alginate, a solution of calcium chloride) through the peripheral nozzle, whereby the surface and periphery of the vehicle polymerize first, thus forcing the suspended cells into the core.

25 In one advantageous application of the invention, the cells are formed by reaggregating natural cell clusters into a form adapted for increased packing per unit volume as described supra.

Such reaggregated clusters are preferably characterized by  
30 improved diffusion of critical solutes to and from the cells within the cluster in comparison to natural cell clusters.

-54-

The newly-formed immunoisulatory vehicle obtained by any of the methods described herein can be maintained under sterile conditions in a non-pyrogenic, serum-free defined nutrient medium or balanced salt solution, at about 37°C, prior to implantation. Lower temperatures (20°C - 37°C) may be optimal for certain cell types and/or culturing conditions. Other holding temperatures and medium compositions consistent with good cell viability may also be used. Alternatively, the vehicle can be cryopreserved in liquid nitrogen, if a cryoprotective agent such as glycerin has been incorporated into the matrix (Rajotte, R.V. et al. (1989) Transplantation Proceedings 21:2638-2640). In such a case, the vehicle is thawed before use and equilibrated under sterile conditions as described above.

Implantation of the biocompatible immunoisulatory vehicle is also performed under sterile conditions. Generally, the immunoisulatory vehicle is implanted at a site in the recipient's body which will allow appropriate delivery of the secreted product or function to the recipient and of nutrients to the implanted cells or tissue, and will also allow access to the vehicle for retrieval and/or replacement. It is considered preferable to verify that the cells immobilized within the vehicle function properly both before and after implantation; assays or diagnostic tests well known in the art can be used for these purposes. For example, an ELISA (enzyme-linked immunosorbent assay), chromatographic or enzymatic assay, or bioassay specific for the secreted product can be used. If desired, secretory function of an implant can be monitored over time by collecting appropriate samples (e.g., serum) from the recipient and assaying them.

The invention will now be further illustrated by the following examples, which are not to be viewed as limiting in any way.

-55-

Example 1: preparation of cells for immunoisolation

Cells derived from cell lines or from primary sources were maintained *in vitro* prior to immunoisolation. (In some cases, cells can be stored cryopreserved and then thawed and acclimated *in vitro*.) Conditions for incubation will vary for specific cell types, but will be readily ascertainable to those skilled in the art following no more than routine experimentation. Islets of Langerhans were obtained by the methods of Scharp et al. (supra) and maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>-95% air in a medium consisting of a nutrient broth (e.g., Ham's F12 (GIBCO)), supplemented with serum (e.g., 25% v/v pooled equine donor serum). Islets were maintained in culture, using Petri dishes at 24°C for predetermined periods of time, according to the method of Lacy, P.E. et. al. (1979) Science 204:312-313. Prior to immunoisolation, the islets were collected, concentrated by swirling the Petri dish, resuspended in Hank's balanced salt solution (HBSS). The washed islets were resuspended in a sufficient volume of HBSS to yield the final islet density required to form an immunoisulatory vehicle containing the desired number of islets, of an appropriate size and shape for implantation and subsequent restoration of normoglycemia to a diabetic individual. This method of preparing the cells prior to immunoisolation is thought to remove antigen presenting cells from the islet tissue thus diminishing immunologic attraction to the outside of the vehicle which could limit its function and duration.

30 Example 2: formation of hydrogel matrices  
with different molecular weight cutoffs

An alginate thin film made from a solution of 1.0% w/v sodium alginate in H<sub>2</sub>O was cross-linked for 6 minutes using either (1) a 1.0% (w/v) or (2) a 2.0% (w/v) aqueous CaCl<sub>2</sub> solution. The sheet was made by placing a film of liquid on a glass plate using a draw down blade with a 0.2 mm clearance, then immersing in the aqueous CaCl<sub>2</sub> solution. A disk was cut from the film using a 47mm

-56-

cutting die. The disk was placed in an Amicon stirred filtration cell and used to filter solutions of several marker solutes under a pressure of 10 psi. The concentration of the marker solute was measured in the retentate ( $C_r$  = average of initial and final retentate concentration) and similarly in the bulked permeate ( $C_p$ ). The rejection coefficient of each hydrogel was calculated as follows:

$$R = 1 - C_p/C_r$$

Thus, a solute which is completely rejected would have a coefficient of 1, and conversely, one which is completely passed through the hydrogel would have a coefficient of 0. The hydrogel resulting from (1) was permeable to 2,000 kD Dextran (Poly Sciences corp.) (rejection coefficient equal to 0.64). The hydrogel resulting from (2) was nearly impermeable to the same Dextran solution. Figure 1 describes permeability of the two hydrogels to the following additional solutes. Bovine Serum Albumin (BSA; ICN Biochemicals), Vitamin B12 (ICN Biochemicals),  $\alpha$ -chymotrypsin (ICN Biochemicals), Apoferritin (Sigma). The approximate molecular weights are given in parenthesis on the Figure.

Example 3: formation of a dual-matrix  
immunoisulatory vehicle

A 2% solution of sodium alginate in physiological saline (PS; 150 mM NaCl) was prepared under sterile conditions. A sterile suspension of pancreatic islets in CRML1066 (GIBCO) culture media isolated from adult rats was diluted 1:1 with the alginate solution, for a final concentration of 1% alginate in the islet suspension. The islet suspension was extruded from a single chamber extrusion nozzle into a 1%  $\text{CaCl}_2$  bath. Once the alginate polyions were crosslinked (approx. 2 min.) and the core containing immobilized islets formed, the core was placed in a 2% alginate solution. The core was then drawn up into tubing with a diameter approximately 500 microns larger than the

-57-

core and reextruded into a second crosslinking bath of 2% alginate, surrounding the core with a jacket formed of a separate cell-free layer of alginate matrix cross-linked to the core. The thus formed macrocapsule was cylindrical with dimensions of 30 mm length, 800 mm core diameter, and 1 mm diameter core to jacket. The core volume was 15 mm. The core contained 300 islets constituting a volume fraction of 10.6% of the total core volume.

10 Example 4: formation of a dual-matrix immunoisolatory vehicle by coextrusion

A 2% solution of sodium alginate in physiological saline (PS; 150 mM NaCl) was prepared under sterile conditions. A sterile suspension of pancreatic islets in CRML1066 culture media isolated from adult rats was diluted 1:1 with the alginate solution, for a final concentration of 1% alginate in the islet suspension.

The islet suspension was loaded into the inner chamber of a nested dual-bore coextrusion device of the configuration described previously, the inner bore of which has a diameter of 500 microns and the peripheral bore of which has a diameter of 600 microns. The outer chamber of this device was loaded with a solution of sterile 1% sodium alginate in PS.

The tip of the nozzle was immersed in a bath containing a sterile solution of 1%  $\text{CaCl}_2$  in PS, which induces the hardening or gelling of alginate by crosslinkage of alginate polyions. The materials loaded into the chambers were coextruded into this bath, generating a continuously forming alginate cylinder containing a core region of alginate matrix-immobilized islets and a surrounding region of alginate matrix free of islets. The outer diameter of the jacket was 1.2mm. The inner diameter of the core was 1.0-1.05 mm. The total islet volume of the core was  $0.8 \text{ mm}^3$  (200 islets). The total core volume was  $25.98 \text{ mm}^3$ . The volume of the islets was 3% of the total

-58-

core volume. The MWCO for 1% alginate was as depicted in Fig. 1. However, the MWCO will increase with time similarly to Fig. 7 due to continued  $\text{Ca}^{++}$  displacement. The alginate of the core was cross-linked with the  
5 alginate of the jacket.

The relative thickness of the surrounding region was modified by adjusting the speeds at which the materials were extruded from the core and peripheral bores of the nozzle. In general as flow in the core increased, wall  
10 thickness decreased. As flow in the peripheral bore was increased wall thickness increased, Ranges of flow rates used were the same for both the core and periphery (0.3 - 1.5 ml/min.). The ends of the cylinder were sealed by dipping the cylinder first in a sterile 2% sodium alginate  
15 bath, then in a sterile 1%  $\text{CaCl}_2$  bath. The immunoisulatory vehicle so formed was maintained in sterile tissue culture medium prior to implantation.

Example 5: Formation of core matrix, permselective membrane immunoisulatory vehicle by coextrusion

20 A suspension of rat islets in 1% alginate was prepared as described in Example 3 and loaded into the inner chamber of the coextrusion device. The outer chamber was loaded with a membrane casting solution comprising 12.5% (w/w) PAN/PVC in DMSO (i.e., dimethyl sulfoxide). The tip of  
25 the nozzle was either positioned at a fixed distance above, or immersed in, a bath containing a sterile solution of 1%  $\text{CaCl}_2$  in PS, which induced the hardening or gelling of the alginate core matrix, and simultaneously induced the hardening of the casting solution into a  
30 permselective membrane. The exterior surface characteristics of the vehicle were determined by whether the nozzle had been positioned above or below the surface of the bath. When the nozzle was positioned above the bath and exposed to low relative humidity (RH) air, an  
35 anisotropic membrane with a rough, coriaceous (i.e., leathery) external surface was formed, but when the nozzle



-59-

was immersed in the bath, a membrane-bilayer with a smooth external surface was formed. Alternatively, when the nozzle was placed above the bath and the fiber exposed to high RH air, an intermediate was formed. The materials loaded into the chambers were coextruded into this bath, generating a continuously forming cylindrical vehicle, comprising a core region of alginate matrix-immobilized islets and a surrounding semipermeable membrane having a MWCO of 50 kD.

10 The relative thickness of the membrane was modified by adjusting the relative velocity of extrusion from the bores, as described in Example 3. The ends of the cylinder were sealed using methods similar to those described in the copending U.S. patent application Serial  
15 No. 07/461,999 filed January 8, 1990, the teachings of which are herein incorporated by reference. The immunoisulatory vehicle so formed was maintained in sterile PS, a balanced salt solution or tissue culture medium prior to implantation.

20 Example 6: Formation of core matrix, permselective membrane immunoisulatory vehicle by "hand loading"  
In other cases, the islets were suspended in a 1-2% alginate solution, and "hand loaded" into preformed thermoplastic hollow fibers using a syringe and the ends  
25 of the fibers were sealed by a combination of heat and polymer glue precipitation as described in copending U.S.S.N. 07/461,999. The MWCO of the thermoplastic jacket was approximately 50 kD. The hydrogel matrix was formed by incubating the loaded fibers in a 1% calcium chloride  
30 solution for 6 minutes.

Example 7: Assessment of viability and function of immobilized, immunoisolated islets in vitro

Adult rat islets immunoisolated within double matrix vehicles by the methods described in Examples 3 and 6.  
35 Matrix liquid core vehicles with thermoplastic jackets

-60-

were incubated *in vitro* for at least two weeks. The vehicle had a core outer diameter of 800  $\mu\text{m}$  overlain with a wall thickness of 65  $\mu\text{m}$ . Alginate/Alginate dual matrix vehicles had an 880 $\mu\text{m}$  core diameter and 60 $\mu\text{m}$  wall thickness. Incubation conditions were: immersion in Ham's F12 medium supplemented with 25% equine serum at 37°C in a 5%CO<sub>2</sub>-95% air atmosphere. The medium was refreshed every three to four days.

Using propidium iodide, the immunoisolated islet cells were found to be 95% viable after this incubation period *in vitro*. They were shown to remain functional as well. When tested by perfusion with glucose (Dionne, *supra*) immunoisolated islets were shown to have an insulin secretory response similar both in magnitude and pattern to that of unprotected islets incubated *in vitro* for a similar period of time and under similar conditions. Insulin release was measured by the method of Soeldner, J.S. et al. (1965) Diabetes 14:771. The results of a typical perfusion experiment are shown in Figures 2A and 2B. The challenge and baseline concentrations of glucose used were 300 mg% and 100 mg%, respectively. No significant delay in either the onset of the first phase of insulin release following glucose stimulation or return to baseline secretion was observed with immunoisolated islets. In addition, a rising second phase comparable to that of unprotected islets was seen. Expressed on a per-islet basis, the total amounts of insulin released by immunoisolated islets were similar to that for unprotected islets. These results are summarized in Figures 3A and 3B.

Example 8: Comparison of *in vivo* performance of xenografted islets isolated within vehicles having a thermoplastic membrane with and without an internal hydrogel matrix

Rat islets were immunoisolated in either matrix or liquid core thermoplastic vehicles as described in Example 6.

-61-

Vehicle dimensions were 800 $\mu$ m O.D., 55 $\mu$ m wall thickness, 2cm fiber length. Just under 20% loading density was used. In the case of liquid core capsules, alginate was not included in the cell suspension. In the first  
5 experiment, islets were immunoisolated within a matrix. Vehicles were implanted intraperitoneally into streptozotocin-induced diabetic mice for a concordant xenograft (i.e., between closely-related species). Free-floating implants were inserted into the peritoneal  
10 cavity. Eight animals were implanted with 500 - 1000 immunoisolated rat islets each. One animal showed no amelioration of hyperglycemia. The others returned to a normoglycemic state (i.e., the plasma glucose levels of these animals returned to a normal range defined as 100-  
15 125 mg%) within five days posttransplantation and remained normoglycemic until day 60 when the grafts were removed and the animals again became hyperglycemic. The average results of 7 such experiments are summarized in Figure 4. The absence of significant fluctuations in plasma glucose  
20 levels in these animals should be noted. The recovered immunoisulatory vehicles were inspected for evidence of fibrotic overgrowth, and were assessed for the ability to release insulin in response to glucose perfusion. None of the vehicles had become completely encapsulated with  
25 fibroblasts, however in some areas three to five layers of fibroblasts around the exterior of the vehicle were observed. Recovered immunoisulatory vehicles released insulin in vitro following perfusion in response to glucose and theophylline stimulation and histological  
30 analysis revealed viable islets with evidence of insulin staining within the  $\beta$  cells. The results of the perfusion experiment with glucose and theophylline stimulation are shown in Figure 5.

These favorable results contrasted sharply with those  
35 obtained when rat islets were immunoisolated within a PAN/PVC membrane without an immobilizing matrix. For

-62-

these immunoisulatory vehicles, functional responsiveness lasted only  $12 \pm 3$  days post implantation; five out of five animals tested returned to a hyperglycemic state thereafter. Histological examination of these

5 immunoisulatory vehicles revealed agglomeration of the islets. The islets had condensed into a large mass of tissue which exhibited severe central necrosis, with only a rim of viable and identifiable islet  $\beta$  cells surviving. Thus, the presence of a matrix to prevent islet

10 aggregation and resulting cell death, significantly improved viability resulting in long term efficacy of the implant.

Example 9: Assessment of the restoration of normoglycemia to streptozotocin-induced diabetic

15 mice by implantation of immunoisolated concordant xenograft islets in immunoglobulin permeable vehicles

*In vivo* performance of the double-matrix immunoisulatory vehicle was assessed using the rat-to-mouse concordant xenograft model for restoration of normoglycemia to

20 streptozotocin induced diabetic mice. The vehicles were prepared as in Example 3 and have significant permeability to 2,000 kD Dextran (Figure 1). Therefore, these vehicles were also readily permeable to IgG (150 kD). The results of this experiment are summarized in Table 2. The animals

25 were divided into three groups: group 1 consisted of seven control animals into whom 300 nonimmunoisolated islets per animal were implanted at the kidney subcapsular site. Only one of these animals showed amelioration of hyperglycemia for more than 12 days. The mean duration of

30 normoglycemia in Group 1 was  $14.0 \pm 3.1$  days.

Group 2 consisted of thirteen animals each implanted with 300 rat islets immobilized in an alginate matrix lacking a surrounding cell-free region. Graft function was lost

35 indicating that simple immobilization conferred no advantage over the Group 1 controls. The other five

-63-

animals remained normoglycemic until the graft was removed. The mean duration of normoglycemia in group 2 was  $29.3 \pm 5.5$  days.

- Group 3 consisted of ten animals, each implanted with 300  
5 rat islets immobilized within a dual-matrix  
immunoisulatory vehicle of the configuration described in  
Example 3, i.e., with a surrounding layer of cell-free  
alginate matrix. In only four of these animals, graft  
function was lost within 14 days postimplantation.  
10 However, six animals remained normoglycemic beyond 100  
days at which time the experiment was terminated and the  
grafts removed, precipitating a return to the diabetic  
state (Figure 6). The mean duration of normoglycemia in  
group 3 was  $65.8 \pm 15.1$  days ( $n=10$ ). Fibromatous reaction  
15 to the recovered alginate vehicle was minimal.  
Histological analysis of recovered immunoisolated islets  
revealed viable islets with evidence of insulin staining  
within the  $\beta$  cells. The immunoisulatory vehicles used in  
this experiment also demonstrate the functionality and  
20 biocompatibility of vehicles which are permeable to high  
molecular weight proteins such as the immunoglobulin G  
protein.

-64-

Table 1

## SURVIVAL OF IMMUNOISOLATED XENOGRAFTS IN DIABETIC MICE

Group	Vehicle	Survival (Days)	
		Individual	Group
5	1	control	7,12,12,12, 12,12,29
	2	immobilized	8,12,12,14, 15,16,18,24, 41*- ,53*,54*, 54*- ,60*-
10	3	immuno- isolated	8,8,12,13, 102*- ,102*- , 102*- ,102*- , 104*- ,104*-
15			

\*Nephrectomy for removal of islet graft

A vehicle of the configuration described in Example 3 was prepared; it contained several hundred islets and had a  
20 membrane MWCO of less than 50 kD.

The vehicle was implanted into a diabetic BB rat. This strain of rat is known to be a rodent model for mimicking human Type 1 autoimmune diabetes. The vehicle was recovered after a 21-day period of residence *in vivo*. The  
25 immunoisolated islets were found to be viable and functional, as determined by histological analysis.

Example 10: Assessment of the survival  
of immunoisolated islets in a discordant  
xenogenic recipient

30 Dual matrix immunoisulatory vehicles containing immobilized rat islets were prepared by the process of coextrusion from a nested dual-bore nozzle as described in Example 4. The conditions for the gelling of the matrix were chosen to yield a hydrogel matrix permeable to 2,000  
35 kD blue Dextran (as in Figure 7), thus the vehicle so formed was permeable to immunoglobulin G and to Clq.

-65-

Segments of about 0.5 cm in length were prepared from the continuous cylindrical vehicle by periodically interrupting the flow of the core material to form cell free regions, which were readily visible. The fiber was cut in the cell free region; thus the cells were completely surrounded by a region of cell-free alginate matrix. The vehicles were implanted between the leaves of the mesentery of guinea pigs (n=2), a discordant (i.e., distantly related) host. After 21 days of residence *in vivo*, the vehicles were removed and tested *in vitro* for glucose-responsive insulin release. These results are summarized in Figure 8. Following basal stimulation, a statistically significant rise in insulin release from the immunoisolated islets was measured when stimulated with 300 mg/dl glucose. A return to basal insulin levels occurred when glucose returned to 100 mg/dl. Thermoplastic vehicles with alginate cores gave similar results.

Example 11: Improved tissue viability  
by controlled reaggregation

Purified canine islets, prepared according to the method of Scharp and Lacy, U.S.S.N. 07/059,125 and U.S.S.N. 07/364,976, were dispersed into cell aggregates containing from one to 50 cells according to the following protocol. 1000 canine islets were rinsed 3 times with 50 ml of Ca++ and Mg++ free Hanks medium containing 1 mM EDTA. After the final rinse, islets were centrifuged into a pellet at 100xg for 8 min., 10°C. The resulting pellet was resuspended in 5 ml of the same medium per 1000 islets. This slurry was agitated for 8 min. at 24°C using a hand held micro-pipet. At the end of 8 min., trypsin and DNase were added to final concentrations of 25 ug/ml and 2 ug/ml respectively. The slurry was further agitated by repeated pipetting for approximately 5 min at which time microscopic examination indicated that the largest aggregates consisted of no more than about 50 cells. The digestion was quenched by adding 10 ml of cold DMEM with 10% new born calf serum per 1000 islets. Aggregates were

-66-

pelleted by centrifugation at 250 xg for 6 min.  
Aggregates were cultured in Ham's F12 with 25% horse serum  
overnight during which time limited reaggregation occurred  
resulting in a volume average aggregate size of  
5 approximately 35  $\mu\text{m}$ .

Following overnight culture, aggregates were pelleted by  
centrifugation at 250 xg for 6 min. A 2% solution of non-  
crosslinked alginate was added to the pellet to make a  
solution consisting of 1% alginate and 8% tissue (w/v).  
10 The final islet volume of the core was  $0.56 \text{ mm}^3$  in the  
form of aggregates evenly dispersed throughout the viscous  
liquid. The tissue slurry was aseptically aspirated into  
a length of Pe 90 tubing the end of which was previously  
necked down so as to fit into the lumen of  $670 \mu\text{m}$  inner  
15 diameter hollow fiber membranes (outer diameter =  $800 \mu\text{m}$ .  
Ten microliters of tissue containing slurry was injected  
into each of several PAN/PVC fibers of 2 cm length. The  
ends of each fiber were sealed as described previously and  
the fibers were stored overnight in Ham's F12 with 25%  
20 horse serum. Prior to implantation, fibers were placed  
into fresh Hanks without serum for 1 hour in order to  
remove serum residue.

After overnight culture, 1/2 of the vehicles were  
implanted into the peritoneal cavity of normoglycemic rats  
25 (2 vehicles per animal) and 1/2 of the vehicles were  
maintained in in vitro tissue culture. After 2 weeks,  
implanted vehicles were removed from the peritoneal cavity  
and subjected to an in vitro glucose challenge along with  
control vehicles cultured in vitro. Explanted vehicles  
30 exhibited insulin release that was as good as or better  
than that seen prior to implantation indicating functional  
survival not only of insulin release but of glucose  
sensitivity. Following glucose challenge, the alginate  
core of the vehicles was "blown out" and the tissue was  
35 stained with PI for viability. Although each aggregate



-67-

had pulled together to form a tight spheroid of approximately 35 um in diameter, individual aggregates remained well distributed throughout the alginate core and had not clustered together to form necrotic regions.

- 5 Failure to stain with PI stain indicated 100% viability.

Example 12: Partial restoration of motor behavior upon implantation of immunoisolated adrenal chromaffin cells in an experimental model of Parkinsonism

- 10 Bovine adrenal chromaffin cells were recovered from adrenal glands according to the method of Livett B.G.. (1984) Physiol. Rev. 64 1103-1161 by collagenase digestion and maintained in culture for 10 days to ensure lack of bacterial or fungal contamination. Clumps of  
15 chromaffin cells were washed in serum-free medium by centrifugation and resuspended in 1% alginate solution. This cell suspension was used to form a matrix-core, thermoplastic membrane immunoisulatory vehicle by co-extrusion as described in Example 4. The aqueous  
20 precipitation bath comprised a solution of 1% CaCl<sub>2</sub> mixed 1:2 with tissue culture medium for a final concentration of 0.5 % CaCl<sub>2</sub> in the bath. The fiber was incubated in the bath for 6 minutes to allow the alginate to gel, and was then transferred to a petri dish containing Dulbecco's  
25 modified Eagle's medium (DMEM). The fiber was visually inspected for regions with good wall morphology, then spot-checked for the presence of chromaffin cells. It was then divided into 4 mm long sections by sealing the ends of the sections with a combination of heat, solvent, and  
30 pressure.

- Eight Sprague-Dawley rats received injections (10µg/5ul) of 6-hydroxydopamine (6-OHDA) into the substantia nigra. They were tested for apomorphine-induced (0.1 mg/kg) rotational behavior at weekly intervals. By the method of  
35 Ungerstedt V.U. (1971) Acton Physiol Scand. Suppl. 367:69-93 and Ungerstedt V.U. (1970) Brain res 24:485-493.

-68-

Apomorphine induces the Parkinson's-like motor response of turning away from the side of the 6-OHDA-induced lesion. The extent of such rotational motor behavior upon apomorphine injection can be used to monitor the extent of the lesion and the degree of amelioration provided by the immunoisolated chromaffin cells. Six weeks after the induction of Parkinsonism, the animals received intrastriatal implants of either control (empty) or adrenal-chromaffin cell-containing vehicles, and were again tested for rotational behavior at weekly intervals. These results are summarized in Figure 9.

Prior to implantation, all animals exhibited an equivalent number of rotations following apomorphine challenge. Within two weeks postimplantation, however, those animals receiving immunoisolated adrenal chromaffin cells exhibited a significant 35-40% percent decrease in rotations which remained stable throughout testing; this indicates that the implants significantly reversed the effects of 6-OHDA-induced lesions. The animals who received control vehicles did not show any reduction in the number of rotations.

Example 13: Prevention of neural damage due to excitotoxicity

This example sets forth a method for prevention of neural damage due to neural excitotoxicity in a subject by implantation of a vehicle containing cells which secrete a trophic factor. This animal model of neuroexcitotoxicity is considered analogous to the types of neural damage suffered by patients with Huntington's disease.

Subjects:

Male Sprague-Dawley rats (N = 23) 90-120 days old and weighing approximately 225-250 grams were used in the following experiments. All animals were housed groups of 2-3 in a temperature and humidity controlled colony room maintained on a 12 hour light/dark cycle with lights on at

-69-

0700 hours. Food and water were freely available during testing.

Preparation of adrenal cell-containing macrocapsules:

Bovine adrenal chromaffin cells were isolated from adrenal glands and maintained in culture for 10 days to ensure lack of bacterial or fungal contamination. Clumps of chromaffin cells were washed in serum-free medium by centrifugation and resuspended in 1% alginate solution. This cell suspension was used as the bore solution for coextrusion in 15% PAN/PVC:DMSO solution. The coextruded fiber containing chromaffin cells was collected in a bath of 1%  $\text{CaCl}_2$  mixed 1:2 with tissue culture medium. The fiber was left in that solution for 6 minutes to allow the alginate to gel, and was then transferred to a petri dish containing medium. Fiber was visually inspected for regions with good wall morphology, then spot-checked for the presence of chromaffin cells. Capsules were made by sealing the ends of 4 mm long sections with a combination of heat, solvent, and pressure. Capsules were then implanted stereotaxically into the brains of Sprague Dawley rats.

Preparation of quinolinic acid:

Quinolinic acid (Sigma Chemical Co) was dissolved in 2N sodium hydroxide and diluted with phosphate buffer at pH 7.2 to a final pH of 7.4 and concentration of 225 nmole/1 ul.

Surgical procedure:

Rats were anesthetized with sodium pentobarbital (45 mg/kg) and positioned in a Kopf stereotaxic apparatus. A sagittal incision was made in the scalp and a hole drilled in the skull for placement of the macroencapsulated adrenal chromaffin cells. Capsules were placed in a capillary tube mounted to the stereotaxic device and lowered to the following coordinates: 0.3 mm anterior to

-70-

Bregma, 3.5 mm lateral to the sagittal suture and 7.5 mm ventral from the surface of the brain.

One week later, the animals were anesthetized and mounted in the stereotaxic instrument prior to intrastriatal administration of quinolinic acid or the phosphate buffer vehicle. Solutions were slowly infused (0.125 ul/minute) using a 10 ul Hamilton syringe) through a hole drilled 0.8 mm lateral to the site of placement of the capsule. The injection site for quinolinic acid was: 0.3 mm anterior to Bregma, 2.7 mm lateral to the sagittal suture, and 5.5 mm ventral from the surface of the brain. A total volume of 1.0 ul was delivered and the injection cannula was left in place for an additional 2 minutes to promote the local diffusion of the perfusate. This procedure resulted in the formation of 3 experimental groups: adrenal capsule/quinolinic acid (N = 8), empty capsule/quinolinic acid (N = 8) , quinolinic acid only (N = 7).

Body weights were recorded on a daily basis for 15 days following quinolinic acid administration.

#### 20 Histology:

Thirty days following quinolinic acid administration, animals were transcardially perfused using a peristaltic pump, with 0.9% saline (50 ml/min) followed by 1% paraformaldehyde/1.25% glutaraldehyde in 0.1 M phosphate buffer (4°C) (800 ml/30 minutes). The brains were then postfixed for 2 hours in the paraformaldehyde solution prior to being placed in 20% sucrose for 24 hours. The brains were then frozen and serially cut on a sliding micotome into 30 um coronal sections. Sections were then processed for cytochrome oxidase histochemistry and adjacent sections were stained for Nissl.

Results: The presence of cytochrome oxidase was considered indicative of metabolic activity, and thus of

-71-

neuronal viability. Nissl staining was used to visualize the cell processes and to assess the general structure of the neural architecture.

In the experimental group that received empty capsules, the striatum was shrunken 20 - 40% as compared with the lesioned animals that had received vehicles containing chromaffin cells. The striatal neurons of the empty capsule group showed lack of metabolic activity as demonstrated by the absence of staining for cytochrome oxidase. Furthermore, all animals showed significant decrease in body weight (Fig. 10).

In contrast, the neurons of the group which received vehicles containing chromaffin cells showed normal staining with both cytochrome oxidase and Nissl, and no loss of body weight.

Conclusion: The neurons of subjects which received chromaffin cell-containing implants were protected from excitotoxic damage caused by quinolinic acid.

Example 14: Treatment of neural degeneration

This example sets forth a method of treatment for animals with a fimbria-fornix lesion. This type of lesion produces neuron cell death and degeneration of post-synaptic neurons and behavioral symptoms indicating deficits in memory and learning. The degeneration of cholinergic neurons produced in this animal model are considered analogous to similar effects seen in Alzheimer's disease in humans.

Surgical procedure for production of the fimbria-fornix lesion: Adult male Sprague-Dawley rats (250-350 g) were anesthetized by intraperitoneal injection of sodium pentobarbitol (55 mg/kg). Unilateral fimbria-fornix lesions were performed by aspiration of the fimbria,

-72-

dorsal fornix, medial part of the parietal cortex, ventral hippocampal commissure, corpus collosum, and overlying cingulate cortex. An implant vehicle as described below was then placed into the ipsilateral lateral ventricle of each animal, oriented perpendicular to the interaural plane.

Preparation of PAN/PVC fibers. Permselective hollow fibers were prepared via the dry jet -wet spinning technique (Cabasso, 1980). A 15% solution of PAN/PVC copolymer in dimethylsulfoxide (DMSO) was extruded through an annular spinneret, with solvent DMSO (for a porous inner surface) or nonsolvent water (for a smooth skinned inner surface) flowing through the bore. The resulting fiber was collected into a nonsolvent water bath, glycerinated, and dried.

Genetically engineered fibroblasts. R208N.8 and R208F rat fibroblasts were a gift of Dr. Xandra Breakefield, Harvard University. R208N.8 fibroblasts were engineered to secrete NGF as follows (Short et al., Dev. Neurosci. 12:34-45, 1990). A retroviral vector was constructed from Moloney murine leukemia virus. It contained the last 777-basepairs of the coding region for mouse NGF cDNA under control of the viral 5' long terminal repeat. The vector also included a dominant selectable marker encoding the neomycin-resistance function of transposon Tn5 under control of an internal Rous sarcoma virus promoter. Transmissible retrovirus was produced by transfecting vector DNA into PA317 amphotropic producer mouse fibroblast cells and by using medium from these cells to infect  $\Psi$ 2 ecotropic cells. Virus from the  $\Psi$ 2 clone producing the highest titer was used to infect the established rat fibroblast cell line R208F, transforming it to R208N.8. Individual neomycin-resistant colonies, selected in medium containing the neomycin analog G418,

-73-

were expanded and tested for NGF production and secretion by a two-site enzyme immunoassay.

Encapsulation of fibroblasts. R208F and R208N.8 fibroblasts were dissociated with Trypsin-EDTA, and  
5 suspended at a density of  $1 \times 10^6$  cells/ $\mu$ l in a laminin containing matrigel resuspended in Matrigel™ or Vitrogen™, and aspirated with a 1 cc syringe into a pre-sterilized PAN/PVC hollow fiber. Fibers were cut to 4 mm length, and the fiber ends were heat sealed with a sterile surgical  
10 cautery.

ChAT Immunocytochemistry. After two weeks, the animals were sacrificed, fixed by transcardial perfusion with cold heparinized physiologic saline and 4% paraformaldehyde in phosphate buffer. The brains were immediately dissected  
15 and postfixed overnight, followed by immersion in 15% and 25% buffered sucrose solutions. Frozen sections were cut at 25  $\mu$ m from anterior to posterior on a cryostat, and all coronal sections were collected onto slides or into phosphate buffer. Representative coronal sections were  
20 processed for immunocytochemistry using a monoclonal antibody to rat ChAT (2.5  $\mu$ g/ml) with the biotin-avidin-DAB method. Sections were mounted and neuronal cell bodies counterstained with cresyl violet. All ChAT-positive cell bodies were counted in the medial septum and  
25 vertical diagonal band region ipsilateral and contralateral to the lesion, between the genu of the corpus callosum and the decussation of the anterior commissure. A significant prevention of ChAT(+) cell reduction was observed in rats receiving R208N.8 capsules.

30 Example 15: Use of an immunoisulatory vehicle to deliver a high-molecular weight product to a recipient

Immunoisulatory vehicles were prepared by hand loading 350,000 hybridoma cells producing an antibody (isotype  
35 immunoglobulin G), specific for tumor necrosis factor

-74-

(TNF) into a 7mm length of a medical grade, olefinic microporous hollow fiber of the kind used for plasmapheresis (Plasmaphan; Enka). The internal diameter of the fiber was 300 microns; its MWCO was about 1,000kD.

- 5 The ends of the vehicle were sealed as described in copending U.S.S.N. 07/461,999. The vehicle was implanted under the renal capsule of a mouse, where it was allowed to reside for 14 days. The vehicle was thereafter recovered and found to contain many cells, over 50% of which were viable as determined by the exclusion of indicator dye (pI). The release of TNF-specific antibody into the serum of the recipient mouse was monitored by ELISA. The results are summarized below:

Table 2

15	<u>Days Post Implantation</u>	<u>Titer of TNF Specific Antibody in vivo</u>
	0	none detected
	1	10
	2	30
20	6	70
	8	100
	11	60
	15	23

- A control immunoisulatory vehicle maintained in vitro exhibited similar antibody release.

\*\*\*\*\*

Example 16: subcutaneous implantation of encapsulated islets

- Preparation of islet containing capsules: Two types of acrylic copolymer hollow fibers, designated Type 1 and Type 2 fibers, were used. Fibers were formed by using a dry-wet spinning technique with a spinneret as described in I. Cabasso, Hollow Fiber Membranes, vol 12 of Kirk-Othmer Encyclopedia of Chemical Technology, Wiley, New York, ed 3, 1980, pp.492-517. The acrylic co-polymer used was poly(acrylonitrile-co-vinyl chloride) ( $M_n=100,000$ ,  $M_w=300,000$  as measured by size-exclusion chromatography; CytoTherapeutics, Inc.) dissolved in dimethyl sulfoxide



-75-

- (12.5% w/w). The acrylic copolymer solution was pumped through the outer tube of the spinneret and water was pumped through the inner tube. Type 1 hollow fibers were extruded into water through an air gap, resulting in a fenestrated outer wall typical of fibers made by a dry-wet spinning technique. The type 2 fibers were made in a analogous fashion, except the air gap was replaced by a humidified atmosphere, resulting in a smooth outer surface.
- 10 Rat islets were isolated from male Wistar-Furth rats as described in Example 1 above. The islets were immobilized in alginate gel and encapsulated into 2-cm type 1 or type 2 fibers, 550 or 1000 islets per fiber, as described in Lacy, P.E., et al., Science 254:1782 (1991).
- 15 Implantation: The encapsulated rat islets were implanted intraperitoneally or subcutaneously in mice made diabetic by the injection of streptozotocin. Non-fasting plasma glucose concentrations were determined three times weekly; the diabetic recipients had concentrations greater than
- 20 400 mg/dl before transplantation. The loading density was 70 islets per centimeter for 1000 islets and 35 islets per centimeter for 500 islets. Twenty-six mice received fibers intraperitoneally, and 26 received fibers subcutaneously. In each group, 14 mice received fibers
- 25 containing a total of 1,000 islets and 12 received fibers containing a total of 500 islets.

Results: The intraperitoneal type 1 fibers induced and maintained normoglycemia for greater than 60 days in seven of nine recipients that received 1000 islets and in all of

30 the recipients receiving 500 islets. None of the recipients of subcutaneous type 1 implants of 500 islets remained normoglycemic for 60 days; three of eight recipients of 1000 islets were normoglycemic for greater than 60 days. Removal of the fibers from these three

-76-

recipients returned the mice to a diabetic state. Transplants of rat islets in the type 2 fibers produced and maintained normoglycemia in the recipient mice in greater than 80% of either the intraperitoneal or subcutaneous sites with either 1000 or 500 islets. The recipients became hyperglycemic again when the fibers were removed at 60 days. Histologic examination of the removed type 1 and type 2 fibers revealed that they were biocompatible.

- 10 Example 17: controlled reaggregation of rat islets  
Rat islets were isolated, dissociated, reaggregated and encapsulated as described in example 11, with the exception that sealed fibers were not exposed to serum in vitro and were held for only 1 hour prior to implantation.  
15 Either two 2 cm long capsules or two 2 cm and one 1 cm capsules were implanted in each rat.

- Islets were dissociated and reaggregated to an approximate size of 35  $\mu$ m. All the reaggregated cells from approximately 500 islets were loaded into each 4-6 cm length capsule. Implanted capsules are capable of maintaining normoglycemia in rats for greater than 60 days.

Example 18: fabrication of flatsheet islet encapsulation vehicles

- 25 Aseptic materials and methods were used in all the following procedures. This included autoclave sterilization, EtOH sanitization, UV sterilization and /or 0.2  $\mu$ m sterile filtration.

- Casting solution was prepared using a monoacrylic copolymer with an average molecular weight of  $10^5$  daltons which was dissolved in a water-soluble, organic solvent. The casting solution was 10.0% w/w polymer in the organic solvent. The polymer was precipitated once under sterile conditions prior to its use to remove any residual

-77-

monomers, oligomers, or any additives placed in the bulk polymer by the manufacturer. This polymer solution was then dried and redissolved in 100% DMSO to form a 10% w/w polymer solution. This solution was passed through a 0.2  
5 um sterile nylon filter and collected under aseptic conditions.

Next the casting solution was uniformly spread using a casting bar over a 1/4" glass substrate at a casting thickness of 125 um. In order to cast the film, the  
10 substrate, held at a 30 degree incline, was moved under the stationary casting bar into the precipitation bath. The level of the precipitation bath was between 1/8 - 1/4" from the casting bar. (The substrate can be any material which prevents premature lifting of the membrane from the  
15 substrate prior to complete precipitation. Simultaneous with spreading, the casting solution was plunged into 24° C water resulting in precipitation of the polymer forming an anisotropic semipermeable membrane, with the permselective layer appearing as a thin skin on the quench  
20 side (away from substrate) of the membrane. The film was left in the bath for four minutes to insure that membrane properties have been adequately established prior to its removal.

The film was then carried through a series of rinses to  
25 remove any residual solvent or toxic residue that may compromise the compatibility of the final product. These rinse baths were composed of solutions that caused no marked physical or chemical modification to the initial membrane. The first post bath consists of water processed  
30 through a Milli-Q purification system and was left to soak for a minimum of 15 min. The material was then removed and placed into a 70%v/v punctilious ethyl alcohol and water solution, which had been 0.2 um filtered, for a minimum of 60 mins and then removed. The final stage was  
35 soaking of the film in two sequential sterile normal

-78-

saline baths with volumes of 2 ml/cm<sup>2</sup> fiber for a minimum of 60 min each.

Results: The final wet-as-cast membrane thickness was between 30 um to 75 um with a hydraulic permeability of 0.475 cc/min/cm<sup>2</sup> at 5.0 psig. Rejection coefficient data indicated that the membrane excluded substances larger than approximately 100,000 daltons.

Encapsulation and implantation: Sterile, cast 10% flatsheet membrane soaking in saline was prepared as described above, and encapsulated islets were prepared as follows: A 6 by 8 inch sheet of wet membrane was folded over on itself with the skin sides facing so as to create a strip of double membrane about 1" wide. The fold was carefully pressed to form a crease. Using a #10 scalpel blade, the 1" wide strip of double membrane was cut off from the rest of the sheet. The double strip was picked up by one end and cut into 1" squares using scissors. The squares were caught in saline as they were cut off. The tip and bottom of each square were connected by the fold comprising one side.

Immediately prior to loading, a square was lifted out and placed on the lid of a 3" diameter polystyrene petri dish previously wet with 1-2 ml of 1% CaCl<sub>2</sub> solution. The membrane was unfolded and each side was floated on the CaCl<sub>2</sub> solution with care taken to assure that the solution did not flow into the tip of the membrane.

Previous to this, islets were allowed to settle into a pellet, and resuspended in 1% ungelled sodium alginate solution (solution was prepared by making a 2% alginate solution in H<sub>2</sub>O and mixing this 50/50 with medium in which islets were cultured). Islet/alginate slurry was gently mixed by stirring and aspirating. Slurry was prepared at the rate of 500 islets in 25ul alginate per sq cm useable

-79-

surface area on a single membrane side. This equates to approximately 125 ul and 2500 rat islets for a 1" sq membrane sheet.

Islet/alginate slurry was aspirated into a 200ul pipet tip  
5 and then evenly spread across the inside of one membrane leaving about 1/8 inch gap along all edges of the square sheet. Spreading was done rapidly as alginate slowly crosslinked due to  $\text{Ca}^{++}$  diffusion through the membrane from the underlying  $\text{CaCl}_2$  solution.

- 10 Once the alginate was sufficiently crosslinked to prevent alginate smearing (approx 1-2 min) the other side of the flat sheet device was folded over to form a tip to the flatsheet sandwich. Care was taken to eliminate air bubbles.
- 15 The two sides of the membrane were sealed using an impulse heat sealer with a 1/8" heating element set on medium heat (temp reached between 80-160°C). Each edge, including the folded one, was individually sealed by activating the heat sealer while pressing down on the 1/8" strip of non-  
20 alginate coated membrane along each edge.

After sealing, the device was soaked in 1%  $\text{CaCl}_2$  solution for 4 min so as to further crosslink the alginate. The device was held in Hank's solution until implantation (within 2 hours).

- 25 Flat sheet was implanted in the peritoneal cavity of a chemically diabetic recipient Wistar-Furth rat by making a midline incision through the skin and into the peritoneal cavity of the anesthetized rat. The flat sheet implant was placed in the cavity by grasping the sealed edge with  
30 smooth forceps and gently laying the device, free floating on top of the gut pile proximal to the peritoneal wall. The peritoneal cavity and skin were closed by suturing.

-80-

Animals were studied for 21 days, at which time the devices were explanted. Blood glucose levels dropped from 375 mg/dl to 150 mg/dl within 4 days of implant and remained there until explant, at which time glucose values  
5 rose to 275 mg/dl (n=2).

Histological examination revealed viable islets immobilized in the alginate layer with less than a monolayer of cells attached to the outside of the membrane.

10

EQUIVALENTS

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other such  
15 equivalents are intended to be encompassed by the following Claims:

-81-

## WHAT IS CLAIMED IS:

1. An implantable immunoisulatory vehicle for providing a biologically active product or function to an individual, the immunoisulatory vehicle comprising:
  - 5 (a) a core comprising living cells dispersed in a biocompatible matrix formed of a hydrogel, said cells being capable of secreting a selected biologically active product or of providing a selected biological function to an individual; and
  - 10 (b) an external diffusional surface jacket surrounding said core formed of a biocompatible hydrogel material free of said cells projecting externally thereof, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological
  - 15 rejection of the cells but permitting passage of substances between the individual and core through said jacket required to provide said biological product or function, said core and external jacket forming an interface substantially free of direct ionic bonding and
  - 20 of an intermediate linking layer.
2. The immunoisulatory vehicle of Claim 1 in which said core and jacket are formed of the same type of hydrogel.
3. The immunoisulatory vehicle of Claim 2 in which said hydrogel comprises alginate cross-linked with a
- 25 multivalent ion.
4. The immunoisulatory vehicle of Claim 1 with a core volume in excess of  $1\mu\text{l}$ .
5. The immunoisulatory vehicle of Claim 1 wherein said vehicle is of sufficient size and durability for retrieval
- 30 after implantation.

-82-

6. The immunoisulatory vehicle of Claim 1 in which said cells are selected from the group consisting of insulin-producing cells, adrenal chromaffin cells, antibody-secreting cells, fibroblasts, astrocytes, Beta cell lines,  
5 and Chinese hamster ovary cells.

7. The immunoisulatory vehicle of Claim 1 in which the cells are derived from a cell bank.

8. The immunoisulatory vehicle of Claim 1 in which all of the cells are disposed at a distance no greater than  
10 about 800 $\mu$ m from a diffusional surface.

9. The immunoisulatory vehicle of Claim 1 in which said jacket is between about 5 and 200 microns thick.

10. The immunoisulatory vehicle of Claim 1 in which the interface between said core and jacket are free of an  
15 intermediate layer of fibrogenic material linking said core and jacket.

11. The immunoisulatory vehicle of Claim 1 in which said core includes at least about  $10^4$  cells.

12. An immunoisulatory vehicle for providing a  
20 biologically active product or function to an individual, the immunoisulatory vehicle comprising:

(a) a core comprising living cells dispersed in a biocompatible hydrogel matrix, said cells being capable of secreting biologically active product or of providing a  
25 biological function to an individual; and

(b) an external diffusional surface jacket surrounding said core formed of a biocompatible material, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological  
30 rejection of the cells but permitting passage of substances between the individual and core through said



-83-

jacket required to provide said biological product or function, said core including at least about  $10^4$  cells.

13. The immunoisulatory vehicle of Claim 12 in which the core biocompatible matrix is formed of an alginate.

5 14. The immunoisulatory vehicle of Claim 12 in which said cells are selected from the group consisting of insulin-producing cells, adrenal chromaffin cells, antibody-secreting cells, fibroblasts, astrocytes, Beta cell lines, and Chinese hamster ovary cells.

10 15. The immunoisulatory vehicle of Claim 12 in which said jacket is formed of a hydrogel material.

16. The immunoisulatory vehicle of Claim 12 in which said jacket is formed of a thermoplastic polymer.

15 17. The immunoisulatory vehicle of Claim 12 in which the interface between said core and jacket is free of an intermediate layer of fibrogenic material linking said core and jacket.

18. The immunoisulatory vehicle of Claim 12 in which all of the cells are disposed at a distance no greater than  
20 about  $800\mu\text{m}$  from a diffusional surface.

19. The immunoisulatory vehicle of Claim 12 in which said core and said external jacket form an interface substantially free of direct ionic bonding.

20. The immunoisulatory vehicle of Claim 12 in which said  
25 external jacket is a non-spherical shape.

21. An implantable immunoisulatory vehicle for providing a biologically active product or function to an individual, the immunoisulatory vehicle comprising:

-84-

(a) a core comprising at least about 100 cell clusters dispersed in a biocompatible extracellular matrix, said cell clusters being capable of secreting biologically active product or of providing a biological  
5 function to an individual; and

(b) an external diffusional surface jacket surrounding said core formed of a biocompatible material, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological  
10 rejection of the cells but permitting passage of substances between the individual and core through said jacket required to provide said biological product or function.

22. The immunoisulatory vehicle of Claim 21 in which the  
15 core biocompatible matrix is formed of a hydrogel.

23. The immunoisulatory vehicle of Claim 21 in which said molecular weight cutoff is above about 100 kD.

24. The immunoisulatory vehicle of Claim 21 in which said cells are formed by aggregating naturally occurring  
20 individual cells or cell clusters into an improved diffusional aggregate form adapted for increased packing per unit volume.

25. The immunoisulatory vehicle of Claim 21 in which said cell clusters are selected from the group consisting of  
25 pancreatic islets, adrenal chromaffin cells and insulin secreting cell lines.

26. The immunoisulatory vehicle of Claim 21 in which said jacket is formed of a hydrogel material.

27. An implantable and retrievable immunoisulatory  
30 vehicle for providing a biologically active product to an individual, the immunoisulatory vehicle comprising:

-85-

(a) a core comprising cells capable of secreting a biologically active product selected from the group consisting of growth factor, trophic factor, or both, said cells being dispersed in a biocompatible material, and

5 (b) a surrounding external jacket of a biocompatible material free of said cells projecting externally thereof, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological rejection of the cells but higher than the molecular  
10 weight of the said biologically active product.

28. The implantable vehicle of Claim 27 wherein said cells secrete nerve growth factor.

29. An implantable and retrievable immunoisulatory vehicle for providing a biologically active product to an  
15 individual, the immunoisulatory vehicle comprising:

(a) a core comprising cells from a cell bank, said cells being capable of secreting biologically active product and said cells being dispersed in a biocompatible material, and

20 (b) a surrounding external jacket of a biocompatible material free of said cells projecting externally thereof, said jacket having molecular weight cutoff below the molecular weight of substances essential for immunological rejection of the cells but higher than the molecular  
25 weight of the said biologically active product.

30. An implantable immunoisulatory vehicle for providing a biologically active product or function to an individual, the immunoisulatory vehicle comprising:

(a) a core comprising living cells dispersed in a  
30 biocompatible matrix formed of a hydrogel, said cells being capable of secreting a selected biologically active product or of providing a selected biological function to an individual; and

-86-

(b) an external diffusional surface jacket of non-spherical shape surrounding said core formed of a biocompatible hydrogel material, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological rejection of the cells but permitting passage of substances between the individual and core through said jacket required to provide said biological product or function.

31. The immunoisulatory vehicle of Claim 30 wherein said vehicle is a flat sheet.

32. The immunoisulatory vehicle of Claim 30 wherein said external jacket is in substantially flat or tubular form.

33. The immunoisulatory vehicle of Claim 30 in which the jacket is formed of a hydrogel.

34. A method of making an immunoisulatory vehicle comprised of a core comprising living cells dispersed in a biocompatible hydrogel matrix, said cells being capable of secreting biologically active product or of providing a biological function to an individual, and an external jacket surrounding said core which is a biocompatible, permselective material free of said cells, comprising coextruding:

(a) a suspension of said cells in a biocompatible hydrogel matrix precursor sufficient to maintain the cells dispersed therein, and

(b) a solution of a biocompatible jacket wall-forming precursor material from a nested dual-bore extrusion nozzle, wherein the suspension of (a) is coextruded from the inner bore and the solution of (b) is coextruded from the outer bore of the nozzle, under conditions appropriate for the formation of a biocompatible matrix or membrane from the solution of (b).

-87-

35. The method of Claim 34, wherein the cell suspension of (a) and the solution of (b) are coextruded under conditions sufficient for the formation of a hydrogel matrix from the hydrogel matrix precursor.
- 5 36. The method of Claim 34, wherein the biocompatible hydrogel matrix of (a) comprises an alkali metal alginate which is coextruded with the solution of (b) into an aqueous multivalent cation solution.
- 10 37. The method of Claim 34, wherein the solution of (b) comprises a hydrogel matrix precursor, and the cell suspension of (a) and the solution of (b) are coextruded under conditions for the formation of a hydrogel matrix from the precursor.
- 15 38. The method of Claim 34, wherein the solution of (b) comprises an alkali metal alginate, and the cell suspension of (a) and the solution of (b) are coextruded into an aqueous calcium chloride solution.
- 20 39. The method of Claim 34, wherein the solution of (b) comprises a water-insoluble thermoplastic polymer or copolymer dissolved in a water-miscible solvent and the suspension of (a) and the solution of (b) are coextruded into an aqueous liquid.
- 25 40. A method of forming an implantable and retrievable immunoisulatory vehicle, the method comprising the steps of:
- (a) forming a core comprising a plurality of cells capable of secreting biologically active product in a biocompatible hydrogel medium, and
  - (b) forming an external diffusional surface jacket
- 30 of hydrogel material around said core in direct contact with said hydrogel medium, said jacket hydrogel being of

-88-

sufficient thickness to prevent cells from projecting externally thereof.

41. The method of Claim 40 in which said jacket is formed by cross-linking of the jacket hydrogel and core hydrogel.

5 42. A method of forming an implantable and retrievable immunoisulatory vehicle, the method comprising the steps of:

- (a) forming a jacket of biocompatible material, and
  - (b) loading said jacket with a core comprising a
- 10 plurality of cells capable of secreting biologically active product, said cells being dispersed in a biocompatible hydrogel medium of sufficient viscosity to substantially immobilize said dispersed cells, said jacket having molecular weight cutoff below the molecular weight
- 15 of substances essential for immunological rejection of the cells but higher than the molecular weight of the said biologically active product.

43. The method of Claim 42 in which said jacket biocompatible material is formed of a hydrogel material

20 and said jacket is formed by extrusion.

44. The method of Claim 43 in which said jacket hydrogel material and biocompatible hydrogel medium comprise alginate.

45. The method of Claim 43 in which said jacket is formed

25 of a thermoplastic material.

46. A method of providing a selected biologically active product to a patient in need thereof, comprising implanting into the body of the patient an immunoisulatory vehicle of the type set forth in Claims 1, 2, 4, 7, 10,

30 12, 16, 17, 25, and 27, wherein the cells secrete said biologically active product, whereby said biologically

-89-

active product is secreted from the immunoisulatory vehicle into the patient's body.

47. A method of providing a selected metabolic function to a patient in need thereof, comprising implanting into  
5 the body of the patient an immunoisulatory vehicle of the type set forth in Claims 1, 2, 4, 7, 10, 12, 16, 17, 25, and 27, wherein the cells provide said metabolic function, whereby the metabolic function is supplied to the patient by the cells within the immunoisulatory vehicle.

10 48. A method of providing a selected biologically active function to a patient in need thereof, comprising implanting into the body of the patient an implantable and retrievable immunoisulatory vehicle for providing said biologically active product to an individual, the  
15 immunoisulatory vehicle comprising:

(a) a core comprising cells capable of secreting said biologically active product and dispersed in a biocompatible material, and

(b) a surrounding external jacket of a biocompatible  
20 material free of said cells projecting externally thereof, said jacket having molecular weight cutoff below the molecular weight of substances essential for immunological rejection of the cells but higher than the molecular weight of the said biologically active product, said  
25 jacket being unexposed to serum prior to implantation.

49. A method for treating neural degeneration in a patient comprising implanting in the patient an immunoisulatory vehicle comprising:

(a) a core comprising living cells which secrete a  
30 growth factor, a trophic factor, or both said cells being dispersed in a biocompatible matrix, and

(b) a surrounding external jacket of a biocompatible material free of said cells projecting externally thereof, said jacket having a molecular weight cutoff below the

-90-

molecular weight of substances essential for immunological rejection of the cells but higher than the molecular weight of the substances secreted from the cells whereby the secreted factor is released from the immunoisulatory vehicle into the patient's body to treat the neural degenerative condition.

50. The method of Claim 49 wherein said neurodegenerative condition is caused by neuroexcitotoxicity.

51. The method of Claim 50 wherein said neuroexcitotoxicity is caused by glutamate or glutamate analogs.

52. The method of Claim 49 wherein said neurodegenerative condition is Huntington's disease or AIDS-related dementia.

53. The method of Claim 49 wherein said neurodegenerative condition is cholinergic neuron degeneration.

54. The method of Claim 49 wherein said neurodegenerative condition is Alzheimer's disease.

55. The method of Claim 49 wherein said cells comprise cells which have been genetically engineered to secrete recombinant nerve growth factor.

56. The method of Claim 49 wherein said cells are selected from the group consisting of adrenal chromaffin cells, Schwann cells, glia cells, or astrocytes.

57. The method of Claim 49 wherein said factor is selected from the group consisting of nerve growth factor, basic fibroblast growth factor, ciliary neurotrophic factor, brain-derived neurotrophic factor, neurotrophin 3, and alpha neuroprotectin.



-91-

58. The method of Claim 49 wherein said biocompatible matrix comprises extracellular matrix components.

59. The method of Claim 58 wherein said components are selected from the group consisting of collagen, laminin, glycosaminoglycans, proteoglycans, fibronectin, elastin, and mixtures thereof.

60. The method of Claim 59 wherein said glycosaminoglycans are selected from the group consisting of hyaluronic acid, chondroitin sulfate, heparin, and heparin sulfate.

61. A method for preventing progression of neural degeneration in a patient comprising implanting in the patient an immunoisulatory vehicle comprising:

(a) a core comprising living cells which secrete a growth factor, a trophic factor, or both, said cells being dispersed in a biocompatible matrix, and

(b) a surrounding external jacket of a biocompatible material free of said cells projecting externally thereof, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological rejection of the cells but higher than the molecular weight of the substances secreted from the cells whereby the secreted factor is released from the immunoisulatory vehicle into the patient's body to treat the neural degenerative condition.

62. The method of Claim 61 wherein said neural degeneration is associated with Parkinson's disease.

63. The method of Claim 61 wherein said neural degeneration is caused by neuroexcitotoxicity.

-92-

64. The method of Claim 61 wherein said neuroexcitotoxicity is caused by glutamate or glutamate analogs.

65. The method of Claim 61 wherein said neurodegenerative  
5 condition is Huntington's disease or AIDS-related dementia.

66. The method of Claim 61 wherein said neurodegenerative condition is cholinergic neuron degeneration.

67. The method of Claim 61 wherein said neurodegenerative  
10 condition is Alzheimer's disease.

68. The method of Claim 61 wherein said cells comprise cells which have been genetically engineered to secrete recombinant nerve growth factor.

69. The method of Claim 61 wherein said cells are  
15 selected from the group consisting of adrenal chromaffin cells, Schwann cells, glia cells, or astrocytes.

70. The method of Claim 61 wherein said factor is selected from the group consisting of nerve growth factor, basic fibroblast growth factor, ciliary neurotrophic  
20 factor, brain -derived neurotrophic factor, neurotrophin 3, and alpha neuroprotectin.

71. The method of Claim 61 wherein said biocompatible matrix comprises extracellular matrix components.

72. The method of Claim 71 wherein said components are  
25 selected from the group consisting of collagen, laminin, glycosaminoglycans, proteoglycans, fibronectin, elastin, and mixtures thereof.

-93-

73. The method of Claim 72 wherein said glycosaminoglycans are selected from the group consisting of hyaluronic acid, chondroitin sulfate, heparin, and heparin sulfate.

- 5 74. An implantable and retrievable immunoisulatory vehicle for providing a biologically active product to an individual, the immunoisulatory vehicle comprising:
- (a) a core comprising cells, derived from a specific pathogen free animal, said cells being capable of
  - 10 secreting a biologically active product, said cells being dispersed in a biocompatible material, and
  - (b) a surrounding external jacket of a biocompatible material free of said cells projecting externally thereof, said jacket having a molecular weight cutoff below the
  - 15 molecular weight of substances essential for immunological rejection of the cells but higher than the molecular weight of the said biologically active product.

75. A method for treating diabetes in a patient comprising subcutaneously implanting in the patient an
- 20 immunoisulatory vehicle comprising:
- (a) a core comprising living cells which secrete insulin, said cells being dispersed in a biocompatible matrix, and
  - (b) a surrounding external jacket of a biocompatible
  - 25 material free of said cells projecting externally thereof, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological rejection of the cells but higher than the molecular weight of the substances secreted from the cells whereby
  - 30 the insulin is released from the immunoisulatory vehicle into the patient's body to treat diabetes.

76. A method for providing a biologically active product or function to an individual, comprising implanting into

-94-

the individual's body an immunoisulatory vehicle comprising:

(a) a core comprising living cells dispersed in a biocompatible matrix formed of a hydrogel, said cells  
5 being capable of secreting a selected biologically active product or of providing a selected biological function to an individual; and

(b) an external jacket surrounding said core formed of a biocompatible hydrogel material free of said cells  
10 projecting externally thereof, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological rejection of the cells but permitting passage of substances between the individual and core through said jacket required to  
15 provide said biological product or function.

77. The method of Claim 76 in which said implantation is performed subcutaneously.

78. The method of Claim 77 in which said cells produce insulin.

20 79. The method of Claim 77 in which said cells are actively dividing Beta cells.

80. The method of Claim 77 in which said cells comprise islets of Langerhans.

81. An implantable immunoisulatory vehicle for providing  
25 a biologically active product or function to an individual, the immunoisulatory vehicle comprising:

(a) a core comprising living cells dispersed in a biocompatible matrix, said cells being capable of secreting biologically active product or of providing a  
30 biological function to an individual; and

(b) an external non-spherical jacket surrounding said core formed of a biocompatible material free of said

-95-

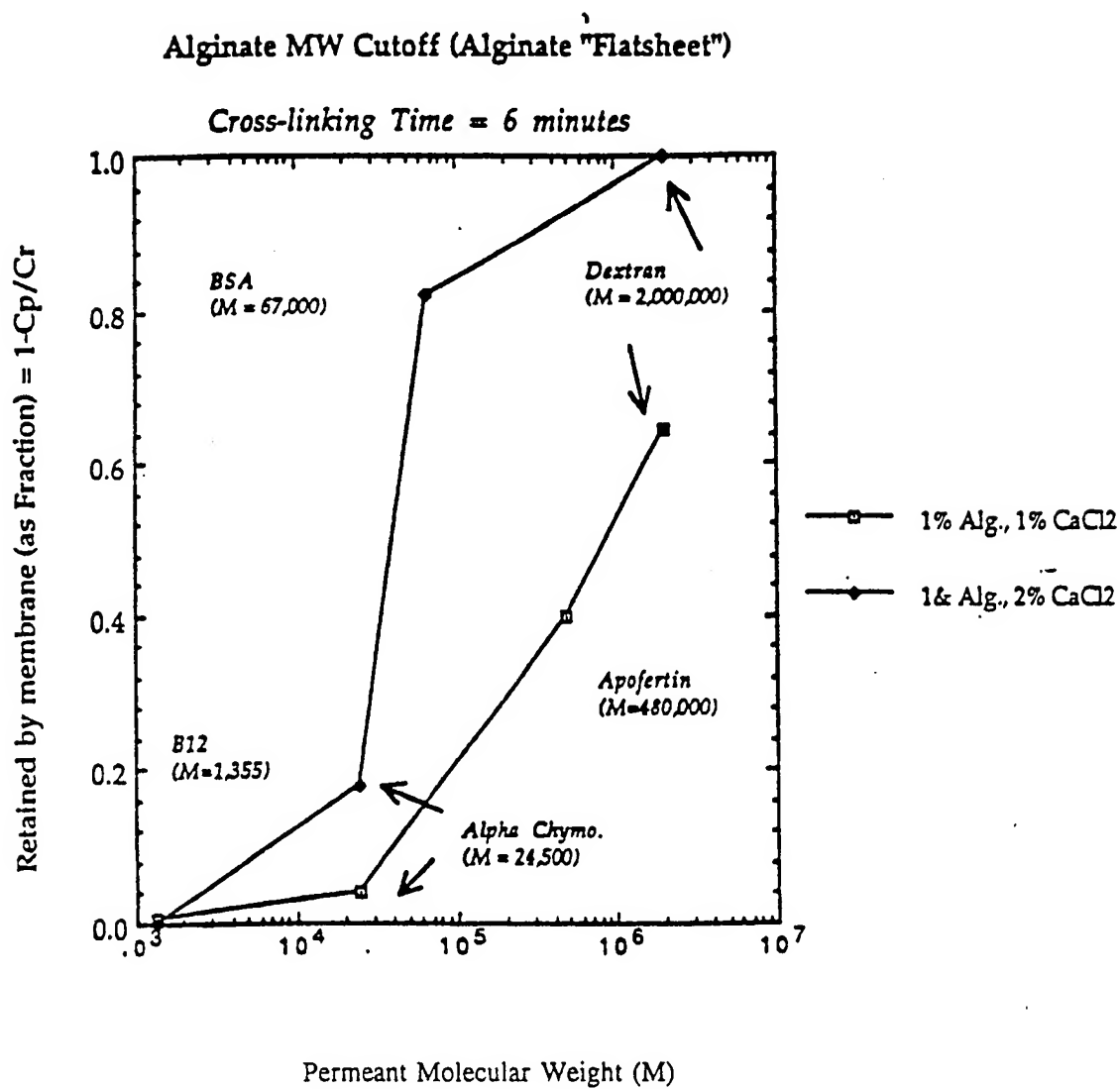
cells projecting externally thereof, said jacket having a molecular weight cutoff below the molecular weight of substances essential for immunological rejection of the cells but in excess of about 100 kD.

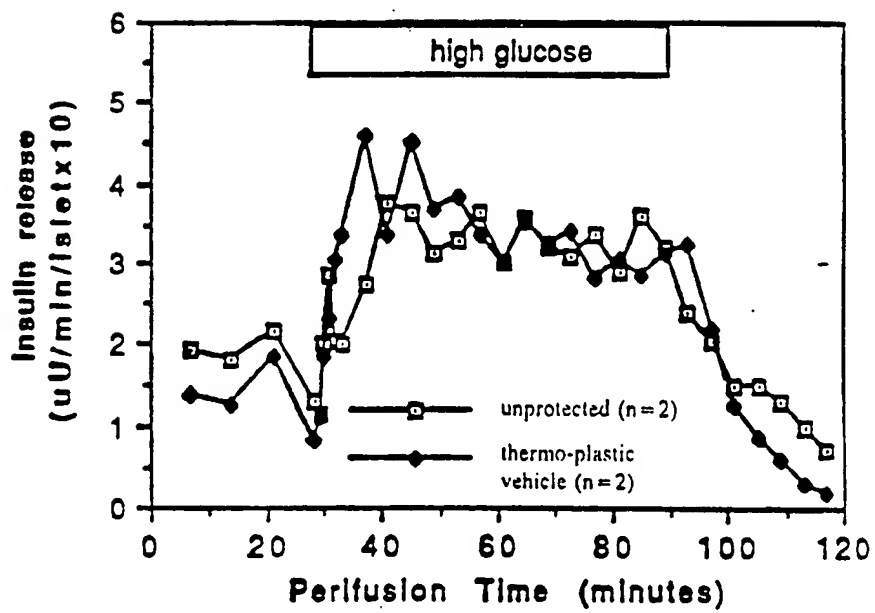
5 82. The immunoisulatory vehicle of Claim 81 wherein said cells are allogeneic to said individual.

83. The immunoisulatory vehicle of Claim 81 wherein said cells secrete antibodies.

1/14

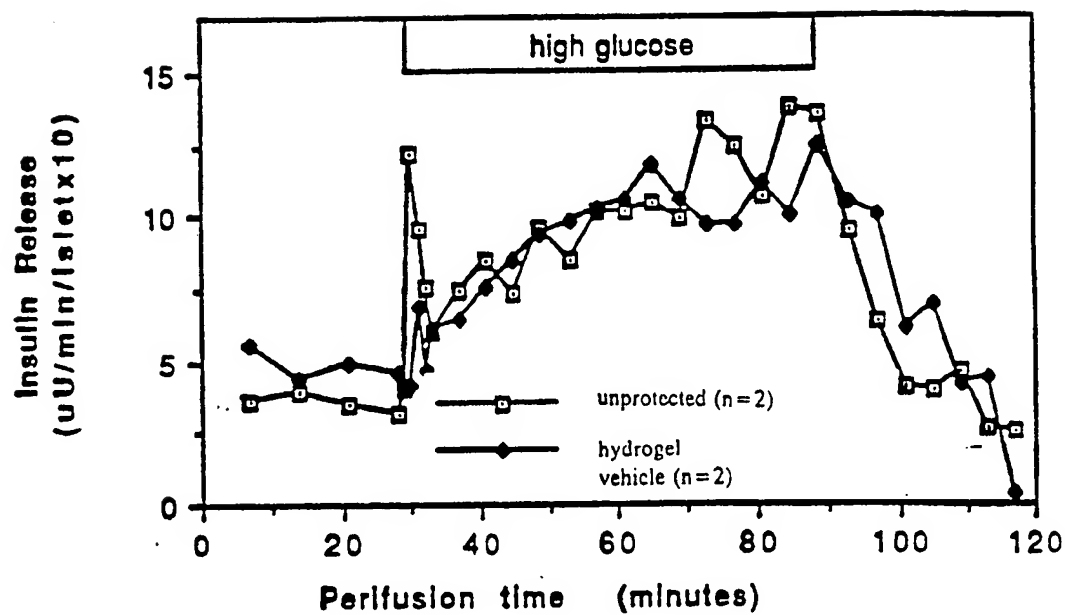
FIGURE 1



2/14  
FIGURE 2AKinetics and Magnitude of Insulin  
Release from Rat Islets

3/14

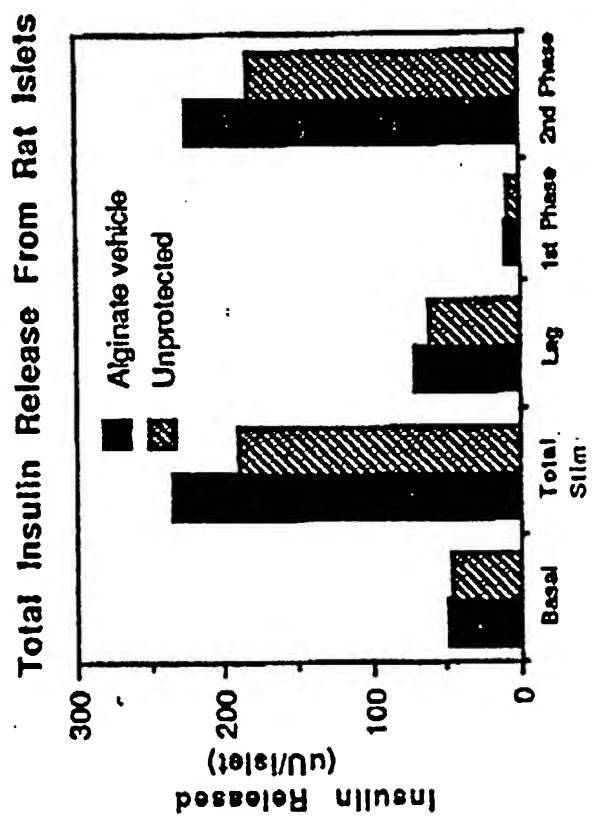
FIGURE 2B

Kinetics and Magnitude of Insulin  
Release from Rat Islets



4/14

FIGURE 3A



5/14

FIGURE 3B

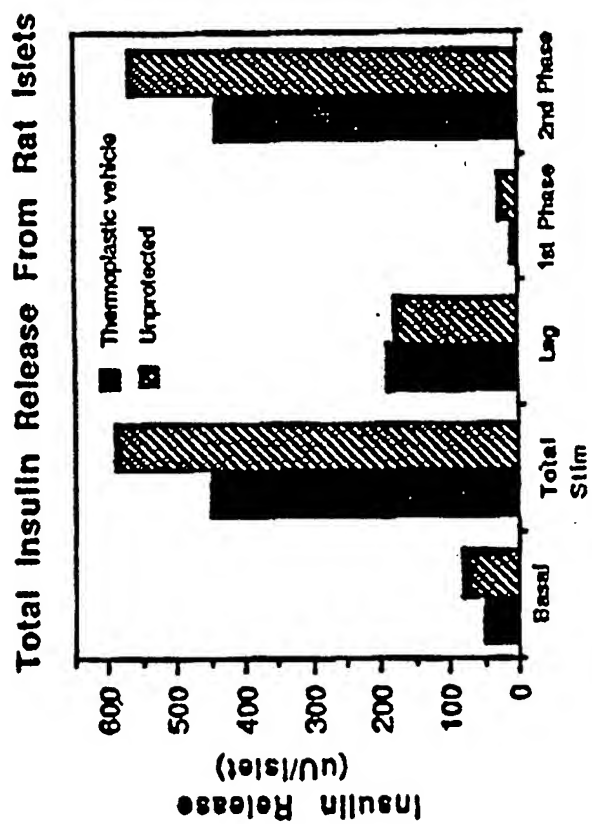
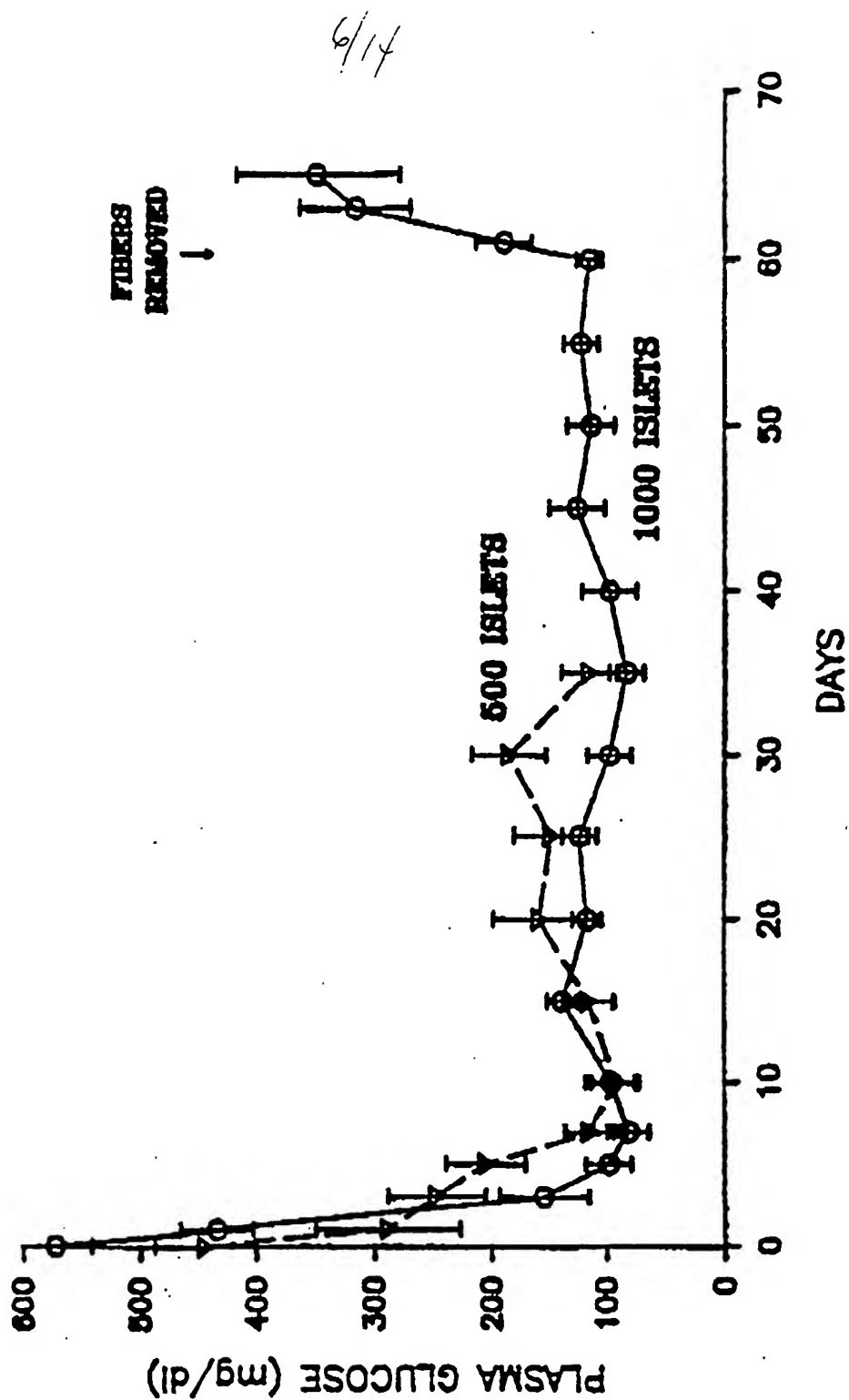


FIGURE 4

RAT ISLETS IN MOUSE N=7



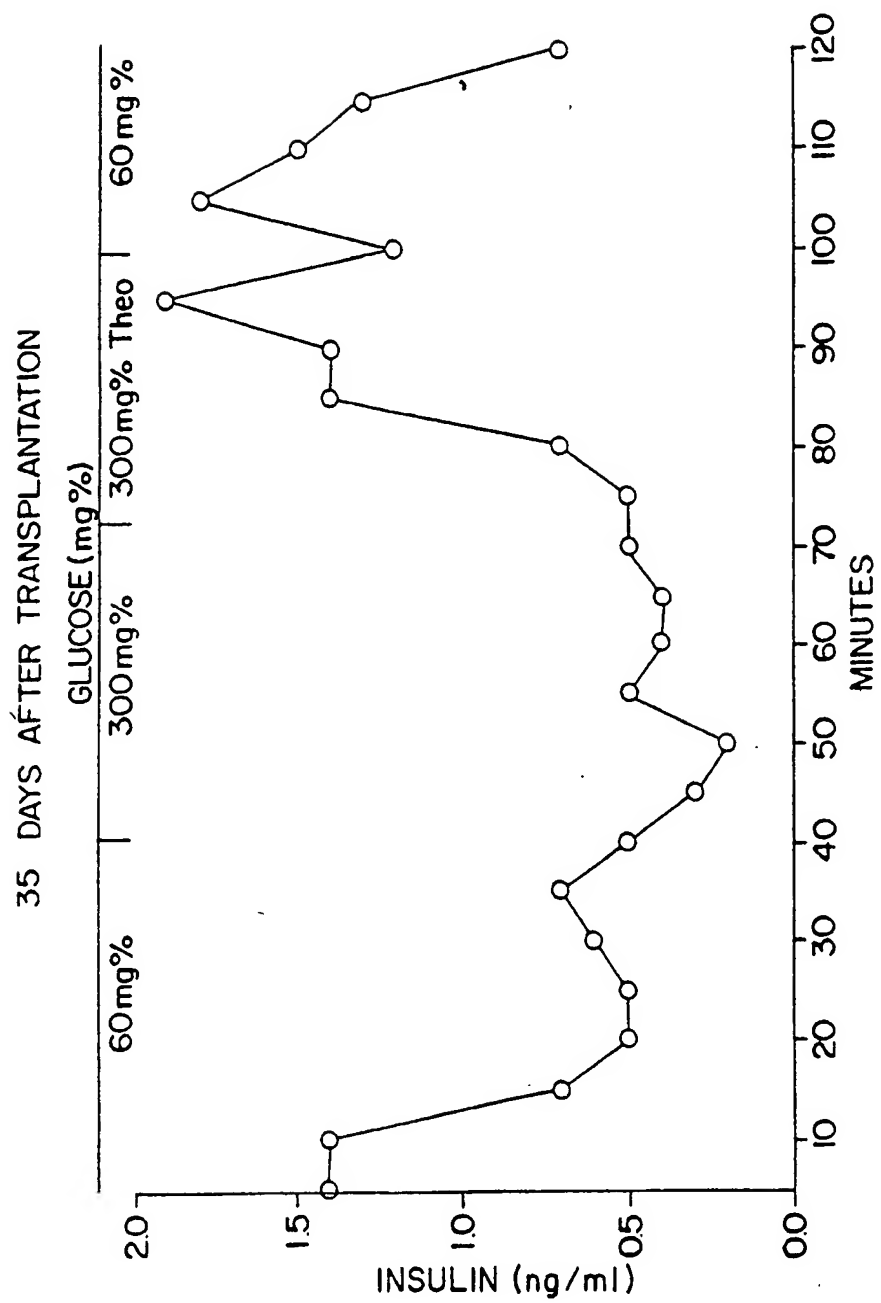
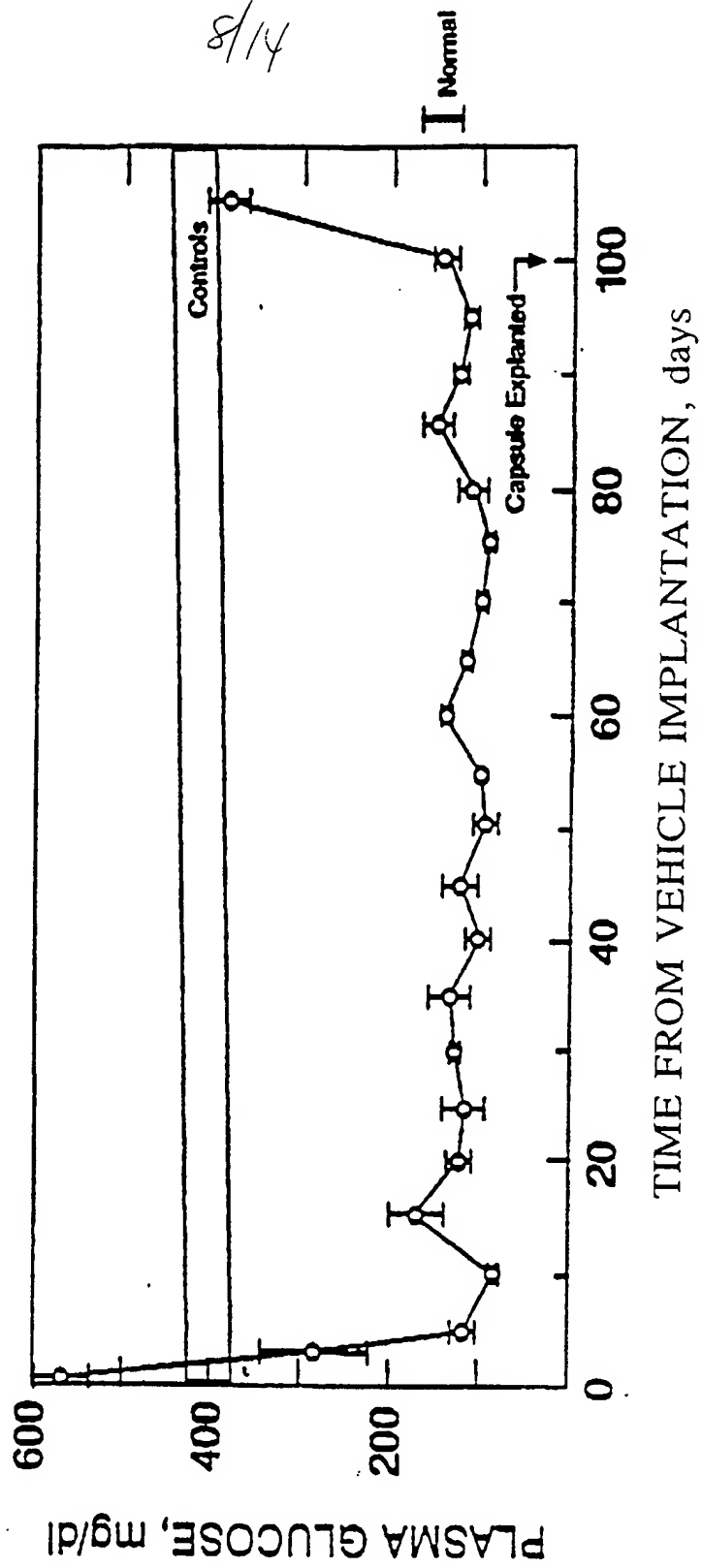
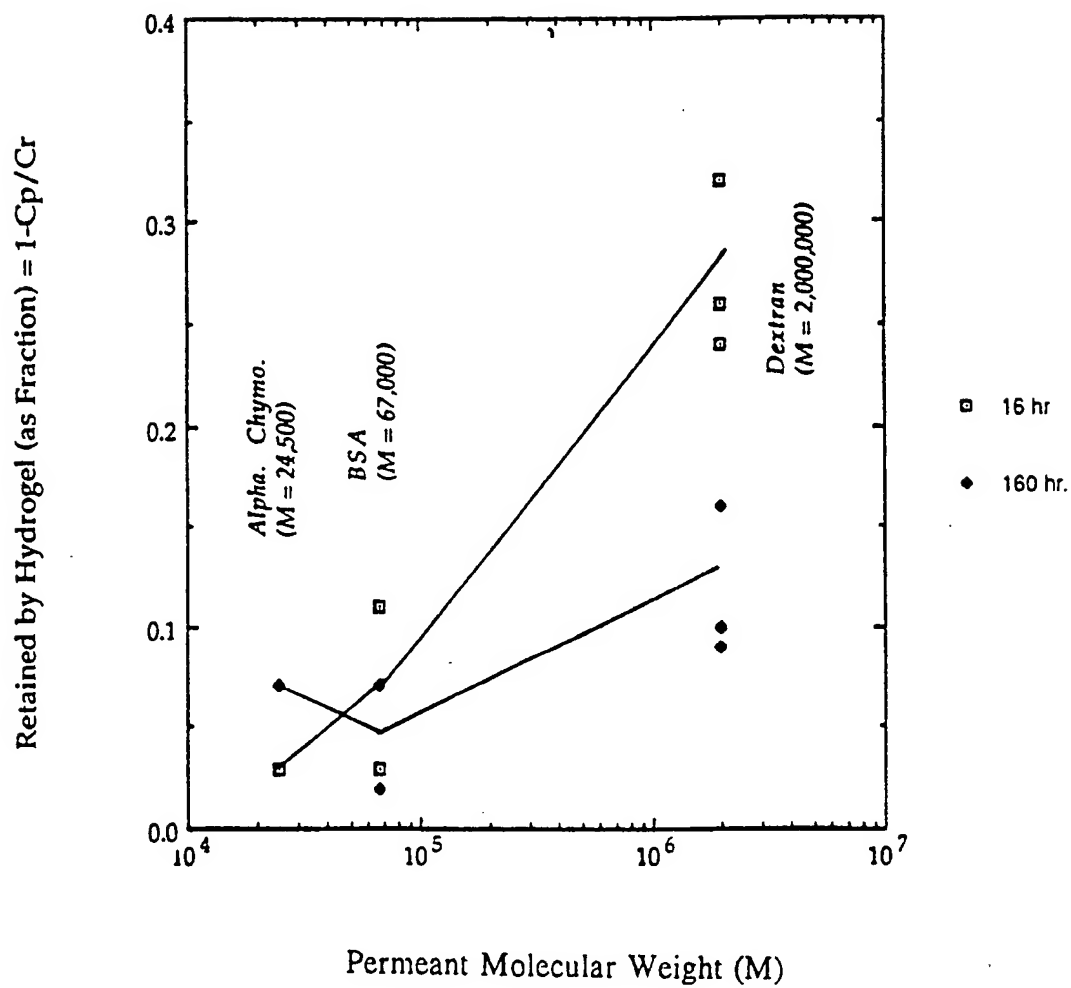


FIG 5

FIGURE 6

**BLOOD GLUCOSE LEVELS  
FOLLOWING VEHICLE IMPLANT  
Rat to Streptozotocin-Diabetic C-57 Black Mouse Implant Under Renal Capsule  
n = 10**

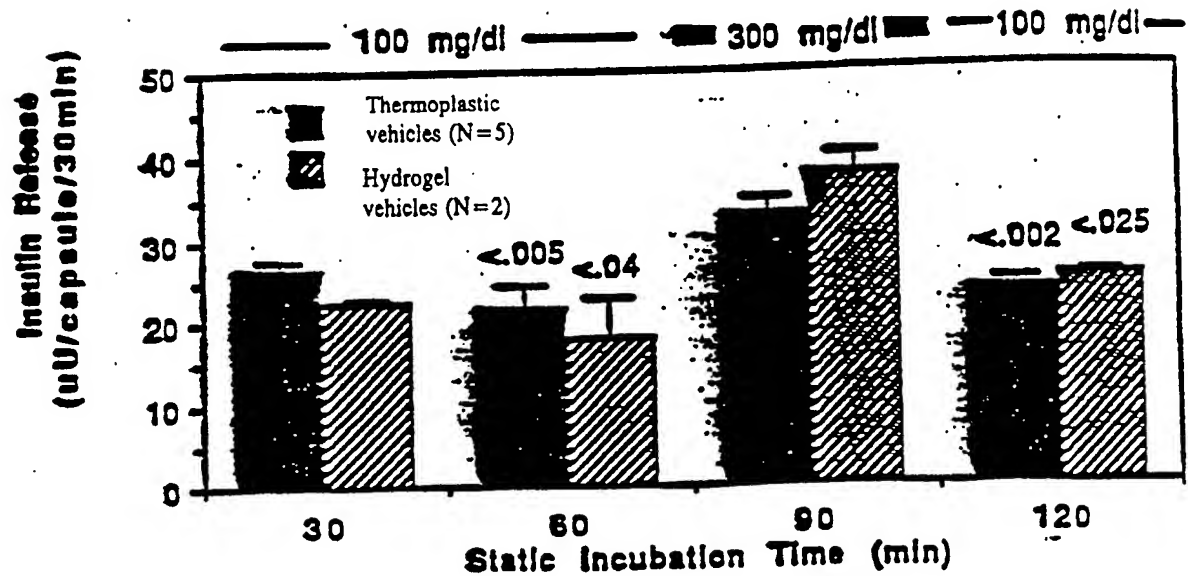


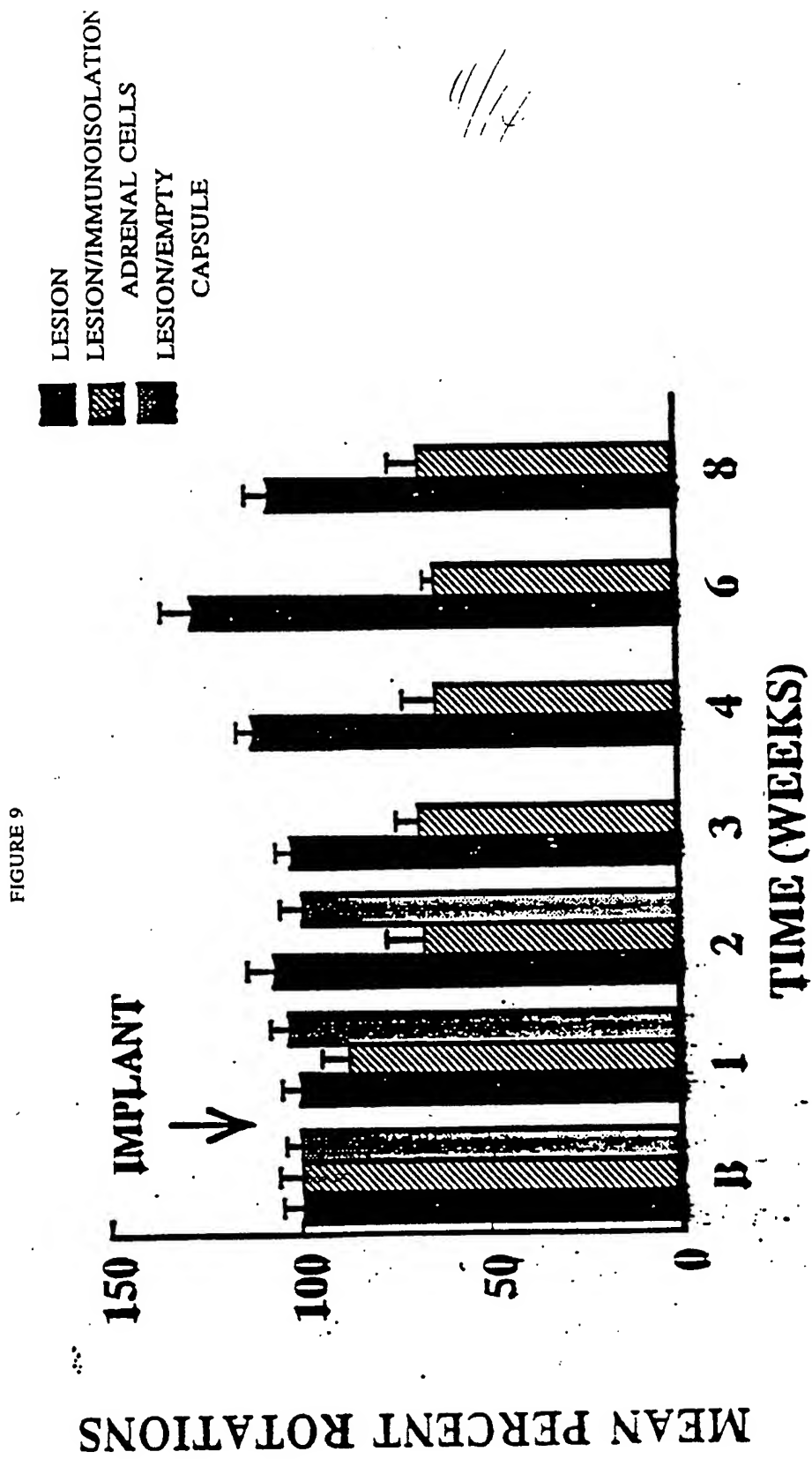
9/14  
FIGURE 72% Alginate / 2% CaCl<sub>2</sub> / 2 min (16 hr. and 160 hr.)

10/14

FIGURE 8

Glucose-stimulated Insulin Release In vitro  
from immunoisolated rat islets 21 days  
posttransplantation to Guinea Pig

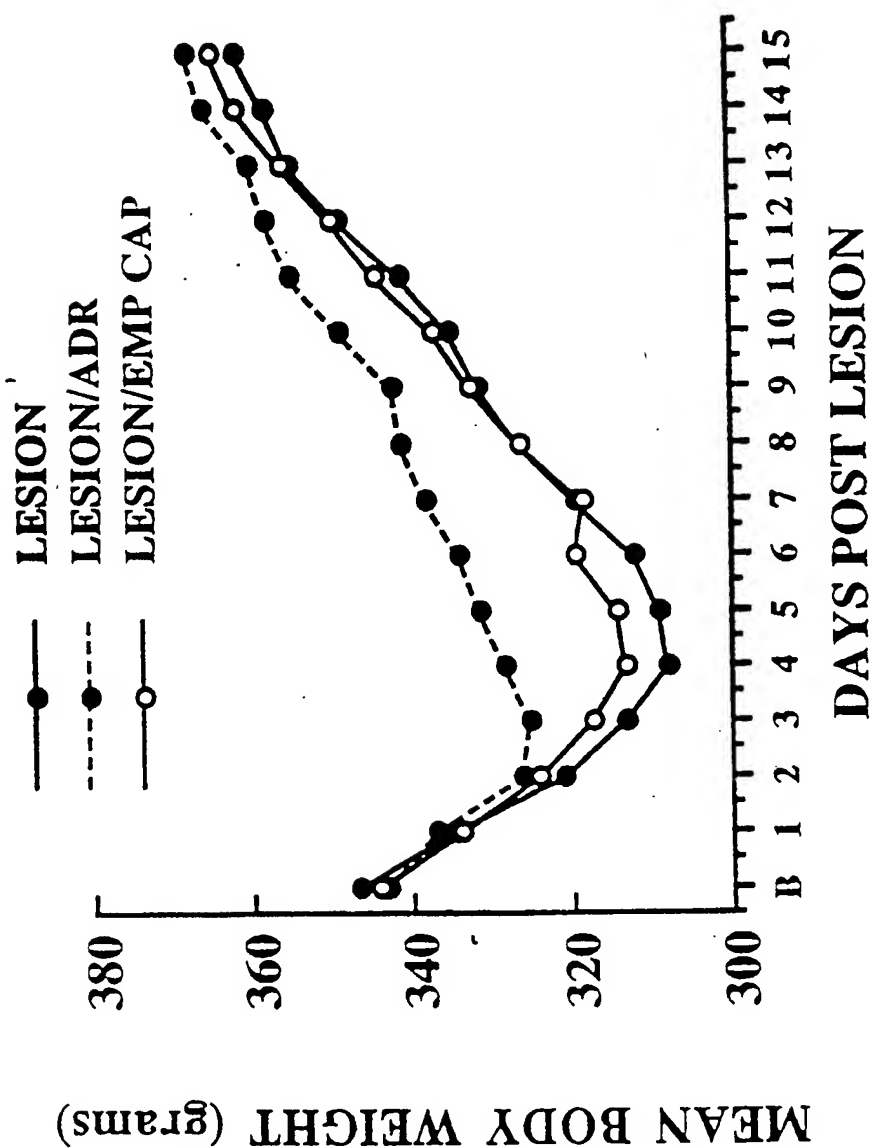






12/14

FIGURE 10



13/14

FIGURE 11

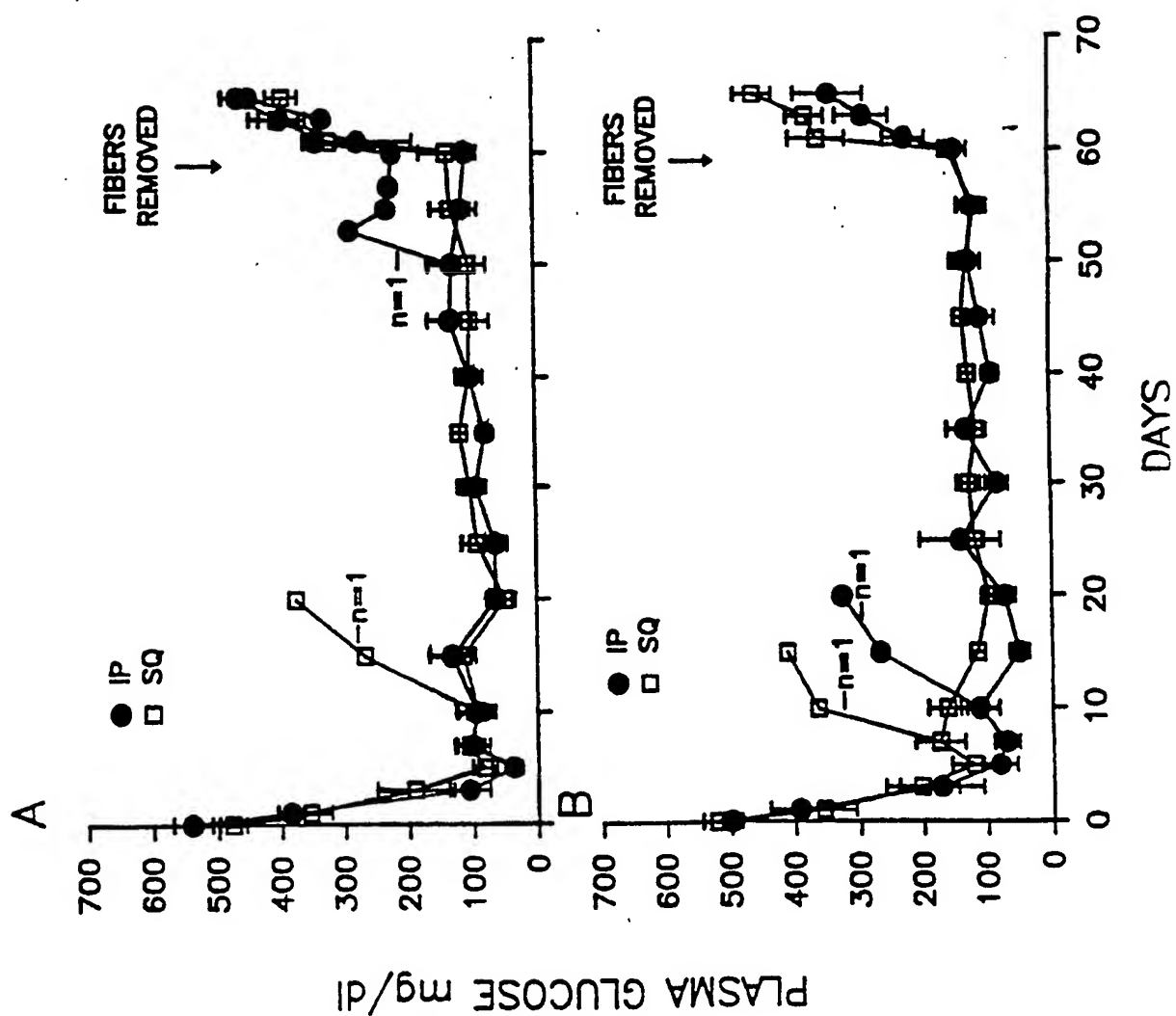
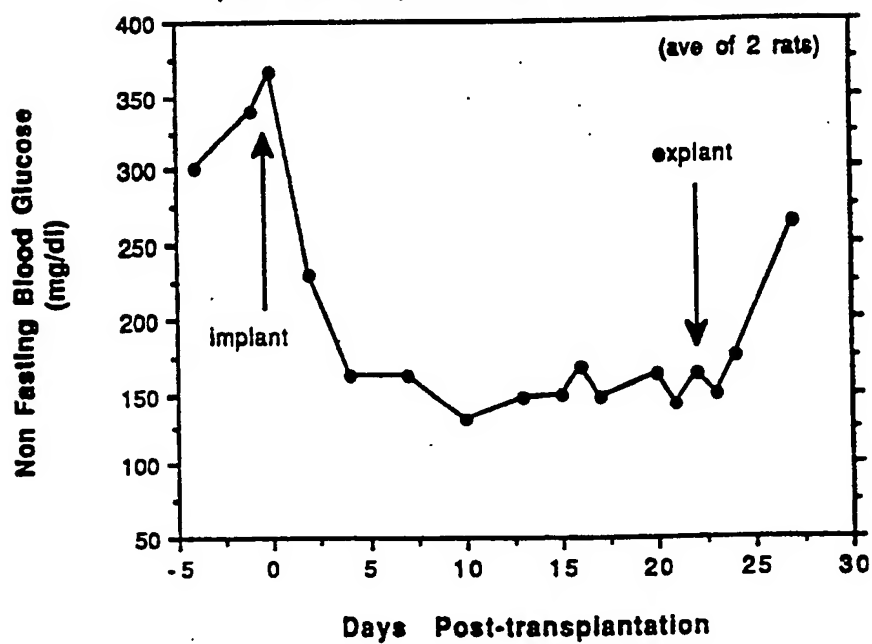


FIGURE 12

14/14

**Flat Sheet Implant Into Alloxanized Diabetic Rats:  
(rat islets; peritoneal cavity implant)**



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/03327

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61F 13/00

US CL :424/422

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/424; 435/240.22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A 4,942,129 (GOOSEN ET AL) 17 JULY 1990 COLUMNS 3-5, 17,18	1-45,48-83
Y	US,A 4,663,286 (TSANG ET AL) 05 MAY 1987 COLUMN 2, LINES 10-25; COLUMN 5, LINES 1-21, COLUMN 6, LINES 23-35	1-45,48-83
Y	US,A 4,353,888 (SEFTON) 12 OCTOBER 1982 COLUMN 7, LINES 4-27	1-45,48-83
A	US,A 4,806,355 (GOOSEN ET AL) 21 FEBRUARY 1989 COLUMN 2, LINES 40-60	1-45,48-83
A	US,A 4,391,909 (LIM) 05 JULY 1983 COLUMN 2, LINES 38-52	1-45,48-83

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

13 JULY 1992

Date of mailing of the international search report

08 OCT 1992

Name and mailing address of the ISA/  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

SALLIE GARONER

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-2351

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/03327

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: (46 AND 47)  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.